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M I C R O S P O R I D I A   H Y P E R P A R A S I T I C

I N

A N O P L O C E P H A L I D   C E S T O D E S

by

A.S.Dissanaike      B.Sc., M.B., B.S.

From The Department of Parasitology,

London School of Hygiene & Tropical Medicine.

A Thesis presented for the Degree of Ph.D.(Science)  
in the University of London.

AUGUST L 1955

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### ABSTRACT

1. A review of the protozoa reported from animal-parasitic helminths is first made.
2. A detailed description is given of the morphology of the microsporidian found in *Moniezia* in this country.
3. This parasite has been shown to be identical with *Nosema helminthorum* Moniez, 1887, *Plistophora helminthophthora* Labbe, 1899, and *Nosema bischoffi* Weiser, 1951. The valid name for this organism therefore is *Nosema helminthorum* Moniez, 1887.
4. A 'pre-metachromatic granule' appearing in the sporont stages, and the metachromatic bodies of the mature spores have been studied from the point of view of their chemical nature.
5. The spore wall of this parasite has been proved to consist of chitin.
6. The mode of emergence of the sporoplasm has been shown to occur in a manner hitherto unobserved in microsporidia.
7. *N. helminthorum* has been shown to infect *Hymenolepis nana* of mice and rats under experimental conditions. In these tapeworms this microsporidian undergoes a similar development to that in the *Moniezia*s, but the spores assume a smaller size.

8. A Taenia saginata of man was also infected with this organism but only early division stages were obtained.
9. Two oribatid mites Xenillus tegeocranus and Ceratoppia bipilis were fed on the spores of N. helminthorum, and the cells of the midguts of these mites became infected. The spores appearing in the mites <sup>were</sup> smaller than those in the mice worms.
10. The significance of the experimental infections has been discussed, and the life-cycle of N. helminthorum has been described from them.
11. The host-distribution and the geographical distribution of N. helminthorum and its host-parasite relationships have been considered. This parasite is shown to be non-pathogenic and the question of its host-restriction has been discussed.
12. A paraneoxenous association between Giardia ovis and Nematodirus fillicollis of sheep has been discussed.
13. Finally a description is given of a new genus of schizogregarine and a new species of microsporidian, from the fat body of the flour beetle Tribolium castaneum. The oöcysts of the schizogregarine were used in some of the experimental infections of Hymenolepis nana.



## PREFACE

Although this thesis is entitled " Microsporidia hyperparasitic in Anoplocephalid Cestodes " it deals first with a general review of the subject of hyperparasitism in helminths, together with a survey of the literature on the protozoan parasites of parasitic helminths. This is followed by a short list of the several helminths that were examined during the course of this work in order to see whether any fresh examples of hyperparasitism could be found. As a result of this survey a Giardia was found in the intestines of several specimens of Nematodirus fillicollis of sheep, and this was studied in greater detail and is included in Part II of this thesis.

In the course of the experimental infections of Hymenolepis nana of mice with spores of the microsporidian in Moniezias it was decided to see if other sporozoan spores could be made to infect this species of worm. Accordingly, oöcysts which were available in large numbers from infected larvae of a culture of Tribolium castaneum sent to Professor Garnham, were fed to mice harbouring H. nana. The sporozoan from which these oöcysts were obtained proved to belong to a new

genus of schizogregarine, and this was therefore studied in detail, the work being incorporated in Part III of this thesis.

Part I    Microsporidia hyperparasitic in  
Anoplocephalid Cestodes.

Part II    Giardia ovis in the intestine of  
Nematodirus fillicollis - a  
paraneoxenous association.

Part III    A New Schizogregarine Triboliocystis  
garnhami n.g., n.sp., and a New  
Microsporidian Nosema buckleyi n.sp.,  
from the Fat Body of the Flour Beetle  
Tribolium castaneum.



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# PART I



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## Chapter I Introduction

There are a large number of examples of parasites living at the expense of other parasites, and this type of association is not uncommon even among the protozoa. Some interesting instances of third and even fourth degrees of such associations are known of among insects, but as Lapage (1929) points out there is obviously a limit to this.

Before going further into the subject it is necessary to define certain terms that have from time to time been used in connection with these associations. For a long time the term hyperparasitism has been employed to represent the association where one parasite lives on or in and at the expense of another parasite. A certain amount of confusion has, however, resulted by the use of other terms such as superparasitism and epi-parasitism in place of hyperparasitism, so that it is necessary to clarify these terms. The term superparasitism was originally proposed by Fiske (1910) to denote the condition which results 'when any individual host' is attacked by two or more species of primary parasites



or by one species more than once.' It was later restricted by Smith (1916) to that type of association where there is a superabundance of parasites of a single species attacking a single host insect. Haviland (1922) substituted the term epiparasite on the grounds that superparasite is hybrid and etymologically equivalent to hyperparasite. However, as Salt (1934) stated, Smith's definition is the one usually accepted. Again Filey and Wallace (1952) have suggested the replacement of the term hyperparasite by epiparasite in cases where one parasite is parasitised by another, their reason being that hyperparasitism more nearly describes instances of excessive infestation by the same species of parasite ; but as we have already seen such an association has by common usage been referred to as one of superparasitism. Furthermore the term epiparasite suggests, and has been used as synonymous with ectoparasite. The term hyperparasite then must be retained as it has for many years been known by entomologists and parasitologists to mean a parasite of a parasite.

#### Review of Protozoa in Helminths

Instances of hyperparasitism among helminths are not wanting as one can appreciate from Dollfus's (1946)

'Parasites des Helminthes' in which he gives a comprehensive account of all types of parasites that have been reported from helminths up to that time. It is not possible in this work to review the whole field of protozoa hyperparasitic in helminths, but an attempt has been made to summarise Dollfus's observations on the protozoa in parasitic helminths and to include the more recent additions to the literature on the subject. This is followed by the main body of this thesis which is a study of "Microsporidia hyperparasitic in Anoplocephalid Cestodes".

The following tables (Tables I, II, & III) suffice to show at a glance the protozoa that have been recorded from time to time from various organs and tissues of acanthocephala, nematodes, cestodes and trematodes. They indicate that while a number of protozoa are not clearly identified, the majority are either microsporidia or flagellates. The former are true hyperparasites whereas the flagellates are as a rule the intestinal parasites of the vertebrate hosts that have secondarily taken up their positions in the intestines of the trematode or nematode parasites of these hosts. Brumpt and Lavie (1936) referred to this as a paraneoxenous type of association,



TABLE I - ACANTHOCEPHALA AND NEMATODA

HELMINTH	HOST	PROTOZOAN	AUTHOR	DATE	REMARKS
<u>ACANTHOCEPHALA</u>					
<u>Echinorhynchus proteus</u> (= <u>Tomphorhynchus</u> sp.)	Fish ?	"Psorospermie" (microsporidian)	Moniez	1879	First figured in 1788 by C.F. Müller, according to Moniez. According to Balbiani(1884) - a Gregarine; According to Labbé (1899) - <u>Gregarina</u> sp.
<u>NEMATODA</u>					
<u>Heterakis callinae</u>	Turkey Poultry	<u>Histomonas meleagridis</u>	Graybill and Smith Tyzzer	1920 1934	That the flagellate is transmitted to avian host via the egg of the worm. Not yet demonstrated in egg
<u>H. callinae</u>	Young turkey	<u>H. meleagridis</u>	Tyzzer	1934	In intestine of worm; non-flagellate form.
<u>H. callinae</u>	Turkey	protozoan(unidentified)	Desowitz	1950	In intestinal epithelium of worm.
<u>Viannella viscaciae</u>	Viscacha	<u>Giardia</u> sp.	Thomson	1925	In intestinal lumen. None in <u>Trichostrongylus</u> sp. found with it
<u>Cooperia oncophora</u>	Young bull	<u>Giardia</u> sp.	Graham	1935	In intestinal lumen.
<u>Hematodirus fillicollis</u>	Sheep	<u>Giardia ovis</u>	Dissanaike	1954	In intestinal lumen. None in <u>Trichostrongylus</u> sp. Only dead forms in <u>Eunostomus</u> .
<u>Synhacia obvelata</u> <u>Aspiculuris tetraptera</u> }	Mouse	<u>Trichomonas muris</u>	Theiler and Farber	1933, 1936	In intestinal lumen of worms.
<u>Synhacia obvelata</u>	Mouse	<u>T. muris</u>	Becker	1933	In egg of worm.
<u>Ascaris mystax</u> (= <u>Toxocara cati</u> )	Cat	"Psorospermie"? Algal spores ? <u>Mucor helminthophthorus</u>	Bischoff Munk Keferstein	1855 1858 1862	Spores 7.7/4.4 $\mu$ } All in " 4.2-5.9/1.7-2.9 $\mu$ . } genital " 4.5/2 $\mu$ . } system. = <u>Nosema helminthorum</u> according to Moniez 1887. = <u>Plistophora</u> <u>helminthophthora</u> according to Labbé(1899). Munk's species at least probably = <u>N. mystacis</u> .
<u>Toxocara cati</u>	Cat	<u>Nosema mystacis</u>	Lutz and Splendore	1908	Spores 4-4.5/2-2.5 $\mu$ . In genital system. Pinto(1925) queried name of host. Wenyon(1926) regarded spores as same as Bischoff's and Keferstein's.
<u>Toxascaris limbata</u> (= <u>L. asonina</u> ?)	Dog	<u>Nosema helminthorum</u>	Skryabin	1923 1927	In genital organs.
<u>Protoanira muris</u>	Mouse	<u>Thelohania reniformis</u>	Kudo and Hetherington	1922	Spores 3-4/1.5-1.8 $\mu$ . In intestinal epithelium.
<u>Dirofilaria immitis</u>	<u>Anopheles</u> <u>maculipennis</u>	Microsporidian ?	Sambon	1926	Sambon only gives a figure with no further details in text.
<u>Ostertagia circumcincta</u>	Sheep	Microsporidian (Unnamed)	Unpublished	1926 ?	In genital system of female worms causing sterility. Worms found by Professor Leiper, or animal recognised as microsporidian by Professor Thomson.
<u>Spironosea affine</u>	Box-tortoise	Protozoan(unidentified)	Chitwood and Chitwood	1938	In intestinal epithelium.
<u>Ascaris lumbricoides</u>	Man	Gregarines ?	Walter	1858	On either side of intestine. Walter thought they may be genital glands. Labbé(1899) = Acephalines.
<u>Oxyuris ornata</u> (= <u>Coenocorca ornata</u> )	Triton <u>lameus</u>	Gregarines ?	Walter	1858	Walter gave no description. Balbiani(1884) called them Gregarines. Labbé(1899) = Acephalines?
<u>Acanemula</u> sp. <u>Pseudocercaria</u> sp. }	<u>Helonellia</u> <u>antoni</u>	Actinomyxidia ?	Steiner	1926	In body cavity of cephalic region
<u>Trichostrongylus</u> <u>per-racilis</u>	<u>Lacopus scoticus</u>	Amoeboid organisms ? Refringent corpuscles ?	Shipley	1909	Shipley found both in <u>Trichostrongylus per-racilis</u> , but Sollfus (1948) thinks that according to diagrams refringent corpuscles were in <u>Trichosoma loncicollis</u> .

and recognised that it could be direct or indirect. Examples of the former are Giardia sp.(viscaciae ?) in Viannella sp. from the viscacha, (Thomson 1925), Giardia sp.(bovis ?) in Cooperia onchophora from a bull (Graham 1935), Giardia ovis in Nematodirus fillicollis from sheep (Dissanaike 1954), Giardia muris in Echinostoma revolutum from an experimental mouse(Callot 1942), Trichomonas muris in Aspicularis tetra- ptera and Syphacia obvelata of the mouse (Theiler and Farber 1933,1936). As examples of indirect paraneoxenous association Brumpt and Lavier recognised protozoa of vertebrate hosts that secondarily have attacked their helminth parasites, examples of which are the microsporidia in trematodes and cestodes of Tropidionotus natrix (vide Tables II and III), and the sporozoan in cercariae and other larval stages of trematodes found in a snail Puccinea sp. (Martin 1935).

An unique type of hyperparasitism is that exhibited by Histomonas meleagridis which though undoubtedly transmitted from one avian host to another via the eggs of Heterakis gallinae has never been actually demonstrated in the egg of the worm. Tyzzer (1934) and Desowitz (1950) have described protozoan hyperparasites in the intestinal cells of H.gallinae (females), and the

TABLE II - CESTODA

HELMINTH	HOST	PROTOZOAN	AUTHOR	DATE	REMARKS
<u>CESTODA</u>					
<u>Taenia expansa</u> (= <u>Moniezia expansa</u> ) <u>Taenia denticulata</u> (= <u>M. benedeni</u> )	Sheep	<u>Nosema helminthorum</u>	Moniez	1879 1887	Spores 5/2.5 $\mu$ . In all tissues and in eggs of worms.
<u>T. expansa</u> (= <u>M. expansa</u> ) <u>T. denticulata</u> (= <u>M. benedeni</u> ) <u>T. bacillaria</u> (= <u>Hymenolepis bacillaria</u> )	Sheep  Insectivore	<u>Plistophora helminthoph- thora</u>  "	Labbé  "	1899	Spores 4.2-5.9/1.7-2.5 $\mu$ . In parenchyma and genital organs.
<u>Moniezia benedeni</u>	Sheep	<u>Nosema bischoffi</u>	Weiser	1951	Fresh spores 6-6.5/ 4 $\mu$ . In the parenchyma of worm.
<u>Moniezia expansa</u> <u>M. benedeni</u> <u>Moniezia sp.</u>	Sheep Buffalo calf	<u>Nosema helminthorum</u> "	Dissanaike	1955	Fresh spores 5.8-6.2/3.3-3.85 $\mu$ . Spores in sections 4.3-5.9/2.3-3.3 $\mu$ . In all tissues but especially in cortex. I consider <u>M. bischoffi</u> a synonym of <u>N. helminthorum</u> .
<u>Hymenolepis nana</u>	Mice; Rats	<u>N. helminthorum</u> (experimentally)	"	1955	Fresh spores measure on an average 5.7/3.2 $\mu$ . All stages of development seen. Spores infective.
<u>Taenia saginata</u>	Man	<u>N. helminthorum</u> (experimentally)	"	1955	Infection only to stages of develop- ent.
<u>Hymenolepis anthraccephala</u> <u>Plerobia reynoldsi</u>	Insectivore ( <u>Blarina brevicauda</u> )	<u>Stempeilia moniezi</u>	Jones	1943	Spores 4-5/ 1.56-2 $\mu$ . in sections In parenchyma near excretory canals.
Larval cestode ( <u>Plerocercoides bancrofti</u> )	Snake ( <u>Tropidonotus natrix</u> )	<u>Glugea ghicli</u> <del><u>Nosema</u></del> ( <u>Plistophora</u> ) <u>ghicli</u>	Guyénot and Neville  Guyénot, Neville and Ponse	1924  1925	Spores 2-2.4/1.5 $\mu$ . In parenchyma of worm and also in tissues of snake.
<u>Eutetrarhynchus ruficollis</u>	<u>Mustelus sp.</u>	Protozoan ?	Pinter	1909	Dollfus thinks it may be a microsporidian.
<u>Catenotaenia dendritica</u>	Squirrel ( <u>Sciurus vulgaris</u> )	<u>Orosporidium charlestri</u> ( <u>Haplosporidian</u> )	Dollfus	1943	In medulla of worm . Causes parasitic castration.



former believed that they were the non-flagellate stages of Histomonas meleagridis . If these are indeed stages of this protozoan on its way to the ovaries of the worm, we can only speculate that they eventually find their way into the eggs of this worm.

A few examples of parasitic castration and sterility are also seen among hyperparasitised helminths. Dollfus (1943) described a supposed haplosporidian , which he provisionally named Urosporidium charletyi , that caused what he called parasitic castration in Catenotaenia dendritica from a squirrel. A Hexamita reported by Hunninen and Wichterman (1936,1938) in Deropristis inflata from an eel, while not producing complete sterility, was found to attack all the reproductive tissues including testes and ovaries of the trematode. The eggs , when heavily parasitised, were found not to develop into the miracidium stage. One other instance of this type is that of a microsporidian in Ostertaria circumcincta from sheep (vide table I) . This has unfortunately not been reported nor has the genus of the microsporidian been identified. Several females of this worm were found without eggs, and a closer examination revealed that the ovaries of these were completely destroyed by the spores of a typical microsporidian as proved by the

TABLE III - TREMATODA

HELICENTH	HOST	PROTOZOAN	AUTHOR	DATE	REMARKS
<u>TREMATODA</u>					
<u>Lepocreadium trulla</u>	<u>Ocyurus chrysurus</u>	<u>Chilomastix</u> sp.	Manter	1930	In caeca of worm; no flagella seen; no flagellates in intestine of host.
<u>Deropristis inflata</u>	<u>Anguilla chrysurus</u>	<u>Hexamita</u> sp.	Hunninen and Wichterman	1936, 1938	In genital system and vitellaria. Passed in eggs. None in <u>zoogonous</u> <u>tubellus</u> present with it.
<u>Echinostoma revolutum</u>	Mouse (experimental)	<u>Giardia muris</u>	Callot	1942	In caeca of worm.
<u>Cercaria gunnisoni</u>	<u>Limnaea stagnalis</u> <u>proxima</u>	"microciliate"	Hurst	1923	Bacillus shaped 4 $\mu$ long.
<u>Diplodiscus temporatus</u> ( <u>-Heraldiascus temporatus</u> )	<u>Rana clamitans</u> (experimental) <u>Acris gryllus</u> (experimental)	<u>Opalina</u> <u>obtrusoides</u>	Hazard	1941	Experimentally only, and temporary
<u>Brachycoelium</u> sp.	<u>Donax trunculus</u> <u>Tellina fabula</u> <u>A. tenuis</u> , <u>A. solidula</u>	<u>Glugeidae</u> ? <u>Pleistophora</u> sp. <u>Pleistophora</u> sp.	Giard Leger Labbé Guyénot and Naville	1897 1897 1899 1925	In large numbers of metacercariae between shell and mantle.
<u>Gymnophallus stricatus</u> (metacercariae)	<u>Donax vittatus</u>	<u>Nosema legeri</u> - <u>Nosema</u> (Pleistophora) <u>legeri</u>	Dollfus Guyénot and Naville	1912 1925	Dollfus thinks his <u>N. legeri</u> the same as the above, and though Guyénot and Naville think it is a <u>Pleistophora</u> he still believes it to be a <u>Nosema</u> (1946).
<u>Leucithodendrium</u> <u>somateriae</u> (metacercaria of <u>Gymnophallus</u> sp.)	<u>Mytilus edulis</u>	Protozoan ? - <u>Nosema</u> sp. ?	Jameson Dollfus	1902 1946	Seen by Dubois (1901) who thought spores form nucleus for pearl after killing worm. In all tissues of metacercariae except suckers.
<u>Distomum linguatula</u> ( <u>-D. linguata</u> according to Pinto (1937)) ( <u>-Glyptothelcis</u> sp. according to Dollfus (1937))	<u>Bufo marinus</u>	<u>Nosema distomi</u>	Lutz and Splendore	1908	Spores 2.0/0.8-1.0 $\mu$ . Free or in cysts in vitellaria.
<u>Echinostoma</u> larvae (rediae, cercariae and metacercariae)	<u>Limnaea limosa</u>	<u>Nosema echinostomi</u>	Brumpt	1922	Spores 5 $\mu$ long. Not found in <u>Tetracotyle</u> also present in rediae
<u>Spelotrema garcini</u> (metacercaria)	<u>Carcinus maenas</u>	<u>Nosema</u> sp. <u>Nosema</u> (Pleistophora) <u>spelotremae</u>	Guyénot and Ponce Guyénot Ponce and Naville	1924 1925	Spores 3.5 / 1.5 $\mu$ .
<u>Encyclometra bolo-nensis</u> ( <u>-E. squibramorum</u> )	<u>Tropidonotus natrix</u>	<u>Glugea encyclometrae</u> - <u>Nosema encyclometrae</u> also <u>Glugea ghigii</u>	Guyénot and Naville	1924 1925	Spores 3-3.5 $\mu$ long. All tissues.
<u>Telorchia ercolanii</u>					Spores 2-2.5 $\mu$ long.
<u>Cercaria pectinata</u> (sporecysts)	<u>Donax vittatus</u>	<u>Anurosporidium</u> <u>pelseneeri</u> - <u>Uresporidium</u> <u>pelseneeri</u>	Caullery and Chappellier Dollfus Guyénot	1906 1925 1943	First discovered by Pelseneer (1895). Causes degeneration of sporecysts.
	<u>Barnes candida</u>	"			
<u>Spelotrema nicolli</u>	<u>Callinectes sapidus</u>	<u>Uresporidium crescens</u>	Turk	1940	
<u>Leclithocladium</u> sp.	<u>Scomber japonicus</u>	<u>Eaplosporidian</u> ?	Kobayashi	1921	In parenchyma of worm.
Larval trematodes	<u>Succinea</u> sp.	Sporezoan ?	Martin	1937	In body and tail of cercariae and in projections of the body wall. Martin considered it a normal parasite of snail adapted to live in cercariae.



demonstration of the extruded filaments from the spores. As no detailed description of this parasite is available it is not possible to determine its genus with certainty.

#### Preliminary survey of Helminths for Protozoa

At the commencement of this work several helminths belonging to the three groups of Nematodes, Cestodes and Trematodes, were examined in order to see whether any further examples of hyperparasitism could be detected. No attempt was made to undertake a comprehensive survey of either vertebrate hosts or of their helminths, but only those helminths that were easily available in large numbers were examined. The helminths were in most instances identified to the species, but in a few, the main groups to which the worms belonged were noted. The table below summarises the helminths studied. Apart from the microsporidian in Moniezia expansa and M. benedeni, and a Giardia found in the intestines of Nematodirus fillicollis no other examples were met with.

Host	Nematode	Cestode	Trematode
Sheep	<u>Ostertagia circumcincta</u> , <u>Haemonchus contortus</u> , <u>Trichostrongylus</u> sp., <u>Nematodirus fillicollis</u> , <u>Funostomum trigonocephalum</u> .	<u>Moniezia expansa</u> , <u>M. benedeni</u> <u>Helicometra</u> .	<u>Fasciola hepatica</u> .
Dogs	<u>Toxocara canis</u> , <u>Toxascaris leonina</u> .	<u>Taenia</u> spp., <u>Dipylidium caninum</u> .	-
Rabbits	<u>Trichostrongyles</u> (from stomach) <u>Oxyuris</u> (from caecum).	<u>Cittotaenia</u> .	-
Pigs	<u>Ascaris lumbricoides</u>	-	-
Rats & Mice	<u>Aspicularis tetraptera</u>	<u>Hymenolepis nana</u>	
Jackdaws	-	<u>Hymenolepis</u> sp. <u>Anomotaenia</u> sp.	-

## Chapter II

### Microsporidia in Cestodes

#### Introduction

The first report of a microsporidian in cestodes was by Moniez in 1879, who found an organism related to that causing 'pebrine', in Taenia expansa which "étaient répandus en quantité prodigieuse par tous les tissus de leur hôte." He also found it in a single specimen of Taenia denticulata but this was found in the company of a T. expansa which was also infected. Later, in 1887, he named this organism Nosema helminthorum and gave the spore measurements as 5  $\mu$  by 2.5  $\mu$ . He did not mention the vertebrate host from which these tapeworms were collected, but it may be assumed that they were sheep. The present names of these worms are Moniezia expansa and Moniezia benedeni respectively, as a careful examination of the literature has revealed\*. Labbé (1899) apparently describing the same organism from Taenia expansa (= Moniezia expansa), Taenia denticulata (= M. benedeni) and T. bacillaris (= Hymenolepis bacillaris) gave the measurements of the spores as 4.2-5.9  $\mu$  by 1.7-2.5  $\mu$ ., but called it Plistophora helminthophthora since "masses plasmiques sporogènes dans les tissus du

\*I am grateful to Prof. F.T.Leiper for helping to clear some doubts on this .



parenchyme formant parfois des vésicules de 20  $\mu$  avec pellicule. " The next description of a microsporidian was by Guyénot and Naville of Glugea ghigii from a larval cestode Plerocercoides pancerii. Guyénot, Naville and Ponse (1925) renamed it Nosema (Plistophora) ghigii. There is an earlier record of a protozoan from Eutetrarhynchus ruficollis by Pinter (1909) which Dollfus (1946) suggests is probably a microsporidian. In 1942 Jones, working on a cytological problem, accidentally came across a microsporidian which he named Stempellia moniezi and described in detail in 1943. This organism was found in two cestodes Hymenolepis anthocephalus and Diorchis reynoldsi from the insectivore Blarina brevicauda. Finally, Weiser (1951) described what he believed to be a new microsporidian from a single specimen of Moniezia benedeni from a sheep in Yugoslavia. He called it Nosema bischoffi and gave the spore measurements as 6-6.5 by 4  $\mu$ .

In the present work I have found a microsporidian in several specimens of Moniezia expansa and M. benedeni from sheep in this country, and in a Moniezia sp. from a buffalo calf in Pakistan\*. As will be discussed

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\* This material and the information on the host were kindly given by Mr. M.M. Sarwar.

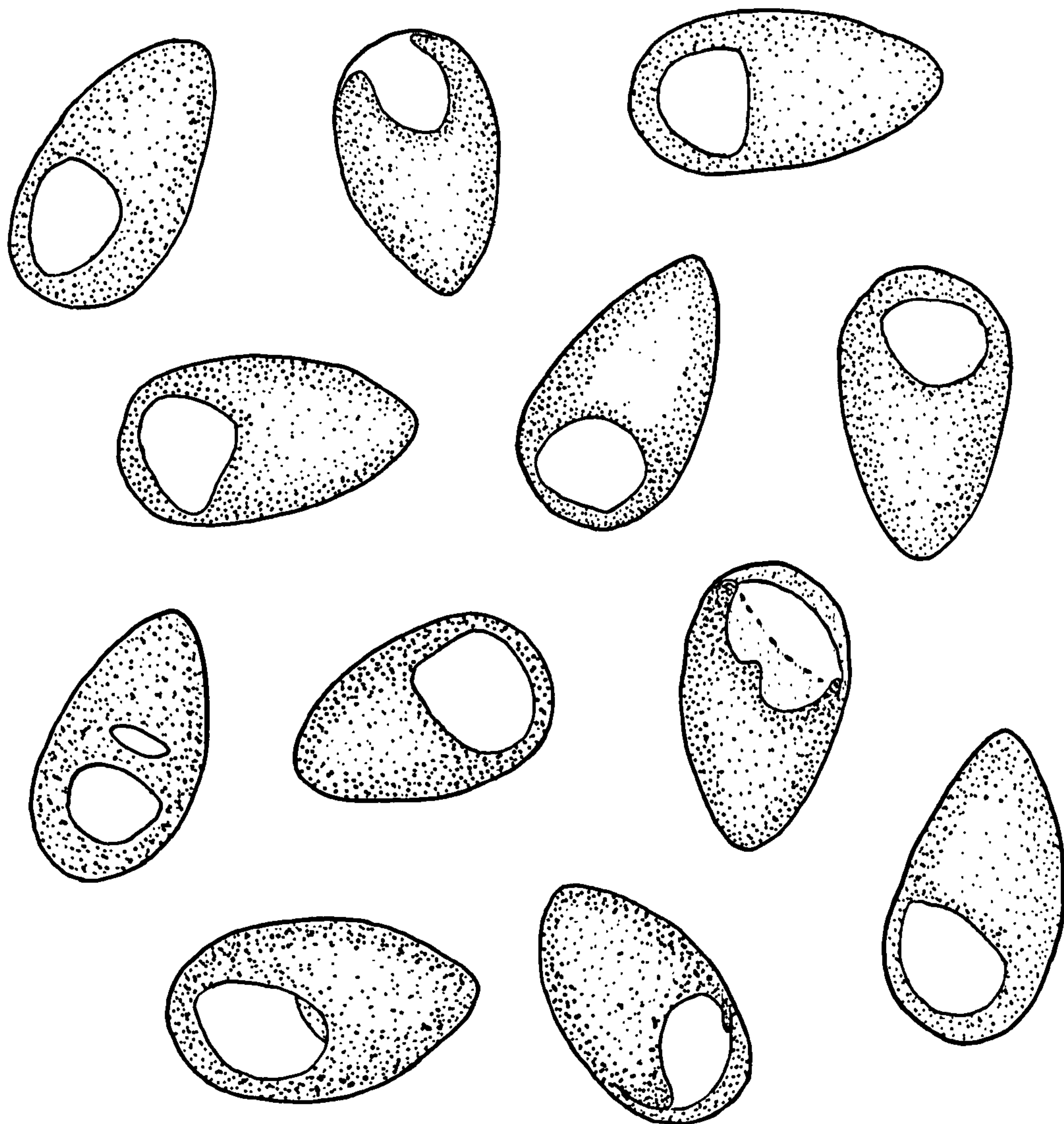
in the next section I regard all these as belonging to the same species, which is Nosema helminthorum Moniez, 1887.

Nomenclature and Synonymy of Nosema helminthorum

It must be conceded that the parasites described by Moniez and by Labbé are the same since they were from the same hosts and had similar sized spores. Labbé put the organism in a separate genus as he saw several spores in cysts surrounded by a pellicle. As will be seen from the description of this parasite it is not uncommon to find this appearance where, due to the breaking down of host tissue, several spores come to lie within a common envelope giving the impression that they are formed from the same pansporoblast. Yet a study of the development leaves no room for regarding this parasite as a Plistophora. As for Weiser's Nosema bischoffi, he distinguishes it from Nosema helminthorum on account of the larger size of its spores. It is my opinion that the measurements of Moniez and of Labbé were made from sections, as their descriptions suggest, and it is quite clear that in sections, spore measurements become very much reduced. Furthermore the spores in the fresh state in my material had the



PLATE I



Fresh spores of Nosema Helminthorum  
from an infected Moniezia expansa .



the following measurements , 5.81- 6.8 $\mu$  by 3.25 -3.85  $\mu$ . which agrees well with the measurements given by Weiser. In the sections of my material the spores as a rule measured 4.3- 5.9  $\mu$ . by 2.3 - 3.3 $\mu$ (Average 5.0  $\mu$  by 2.8  $\mu$ .) in M.expansa and M.benedeni of sheep, and 4.65- 5.7  $\mu$ . by 2.45 - 3.2  $\mu$ . (Average 5.2  $\mu$ . by 2.8  $\mu$ . ), in the Moniezia sp. from the buffalo calf. In addition the spores in the section material of Weiser's N. bischoffi as measured by me were 4.0 - 5.5  $\mu$ . by 2.46 - 3.09  $\mu$ . ( Average 4.5  $\mu$  by 2.7  $\mu$ . )\*. Another important point is that even in the present material I have found considerable variation in the size of spores depending on various factors which will be discussed later. All these facts indicate that the size of the spores alone is not an important criterion in separating species , and final proof of ~~this~~ comes from the experimental infections of other tapeworms and of two oribatid mites with this microsporidian. In these the spores assumed a smaller size though otherwise similar in appearance to those of N. helminthorum . From all this it must be concluded that all the species recorded from various species of Moniezia are the same and that the valid name is therefore Nosema helminthorum Moniez, 1887.

\* I am grateful to Professor Jaroslav Weiser for kindly sending me his material for comparison.

### Material and Methods

The *Koniezias* studied in the present work were collected from sheep slaughtered in the Metropolitan Cattle Market, Islington. They were put into bottles of normal saline and brought to the laboratory for further study. The worms were then sorted out in several petri-dishes, only worms complete with scolices being used especially for working out the incidence of infection. The worms were then examined for evidence of infection by crushing segment by segment starting from the posterior gravid segments. It was noted that when infection was heavy the posterior segments were more heavily packed with spores, so that this was the quickest way to spot an infected worm. Once an infected worm was detected, the distribution of the infection throughout the length of the worm was noted. The infected worms were then identified as far as possible by pressing a few segments between two slides in order to see the appearances of the inter-proglottidial glands. When specific identification was not possible by this method, a few segments were pressed and fixed for staining as whole mounts. When this method too failed horizontal sections of the worms were made

to ascertain the arrangement of the glands.

Studies on Fresh material - The spores obtained from infected worms were first studied in the fresh state in saline, in which preparations the measurements were made. Attempts at extruding the filaments of the spores were made using the standard methods described for the microsporidia. It was found that leaving a suspension of spores in saline over-night with the cover-slip sealed to prevent evaporation, or by using a modified hanging-drop method used by Nemezeck (1926) for the myxosporidia, gave a very high percentage of spores with extruded filaments. In the latter method a cavity slide was used and the spore suspension in a small drop was placed on a 1/4 inch circular cover-slip. This was then covered over with a large square cover-slip and the two placed, with the small cover-slip facing the cavity of the slide. The edges of the large cover-slip were then sealed off with some vaseline.

The fresh spores were further studied by examining them in a very dilute solution of Toluidine blue. After extrusion of the filaments the spores were studied under the dark-ground microscope which enabled the filaments to be seen more distinctly.



Measurements and drawings of fresh spores were made with the aid of a camera-lucida. It was necessary to stop the brownian movement exhibited by the spores in saline suspensions by preparing the slides beforehand with a thin film of agar-agar so that the spores adhered to the agar film. This greatly facilitated drawings and measurements.

Staining methods - For more detailed study of the parasite, dry and wet smear preparations were made, the former fixed in Schaudin's Fluid and both preparations stained in Giemsa stain for 1/2 to 1 1/2 hours. Attempts at staining the wet preparations in Heidenhain's iron haematoxylin were unsatisfactory as mature spores showed many artefacts, an observation that has been made by Jirovec (1936) for other microsporidia. The wet-fixed preparations were on the whole less useful for details of structure. The best preparations of the parasite were obtained by sectioning infected worms that were fixed in one of the following fixatives, and stained by the Giemsa-Colophonium technique:- Carnoy's Fluid, Bouin's Fluid, Schaudin's Fluid, Formo-saline, and a mixture of equal parts of sublimate alcohol and sublimate acetic acid (Jirovec 1936).

The other stains used were Heidenhain's iron

haematoxylin, Mallory's Triple stain, and Maximow's stain.

The Feulgen stain was tried out on sections, using the standard technique modified by Jirovec(1936) for the microsporidia. In this method material fixed in Carnoy's Fluid or Sublimate alcohol/ Sublimate acetic was hydrolised in N.HCl for 4-6 minutes at 60°C. These preparations were counterstained in 1% Light green. Feulgen reactions tried out on smear preparations were unsuccessful.

Cytochemical and Chemical tests- Unna-Pappenheim's methyl green-pyronin stain was used on dry smears to help elucidate the nature of the extra-nuclear acidophilic granule that was present in the sporont stages. The metachromatic bodies occurring in the mature spores were studied by using several stains for metachromatic structures such as 0.5 % Toluidine blue and examining the sections in glycerine jelly. Many other stains were used in an attempt at elucidating the chemical nature of these metachromatic bodies. Many of these were tried out at the suggestion of Dr. A.G.Everson Pearse of The Postgraduate Medical School, London, who was kind enough to carry out most of them for me and give his opinion on the results.

The following reactions were tried out- The Feulgen nuclear reaction, the Unna-Pappenheim stain, the Hotchkiss Periodic Schiff test ( which was repeated using Hale's (1953) modification by pretreating with 0.2 N NaOH to enhance the red stain), the Sudan Black test for fats, Best's test for Glycogen, Millon's test.

In order to test the chemical nature of the spore wall of Nosema helminthorum, various tests for chitin were used as the previous work of Kudo(1921a) and Koehler (1921) had suggested that the spore wall of microsporidia was probably of chitin. The resistance of the spore wall to various dilute mineral acids and their solubility in concentrated acids were verified. The Schultz's and Bethe's tests for chitin were tried out on sections after softening over-night in diaphanol but with negative results even with control sections of arthropod tissue. It was then decided to try Campbell's modification of the van Wissenligh's test. This is not a histochemical test but a destructive one as the relationships of the spores to the other structures around are not maintained, but as Richards (1951) says " the chitosan colour test is the most reliable known today ". The procedure adopted was as



follows- A large test-tube was fitted with a tightly-fitting rubber cork through which a hole was made for a short glass tube of 1/2 cm. diameter. To the end of this tube was fitted a 2-inch-long rubber tubing. The other end of this tube was closed off by a solid glass rod. The rubber tube between the end of the glass tube and the glass rod was slit lengthwise for about 1/2 an inch to function as a bunsen-valve. Into this test-tube was now added about 3-4 c.c. of KOH saturated at room temperature, together with about one inch of heavily infected segments of Moniezia expansa. The tube was now placed in a glycerine bath and heated to 165°C. Once the temperature reached this level it was maintained by removing and inserting the bunsen burner at intervals. The boiling over of the KOH before this temperature was reached by the bath, was prevented by the use of the bunsen-valve as devised by Campbell(1929) in her original method. After the tube was heated in this way for 20 minutes to half an hour, the KOH solution was cooled to room temperature. This now contained as undissolved material such structures as were unaffected by this violent treatment with alkali. The solution was now centrifuged, and washed in turn in 90 %, 70 %, 50 %, 25 % alcohol and then in water, being

centrifuged after each of these washes, and finally a drop of the sediment was placed on a slide and covered over with a cover-slip. This was then flushed with a few drops of 0.2 % Iodine in 1 %  $H_2SO_4$ . A characteristic rose-red or purple colouration indicated that the substance was chitosan sulphate, which on addition of 3 % Acetic acid, dissolved proving that the original substance was chitin. A piece of cockroach cuticle was similarly treated as a control.

Experimental procedures - In the experimental infections using the spores of N. helminthorum the following methods were employed- In most instances the spores were introduced by merely feeding heavily infected segments of Moniezia to the various animals used. In the experiments with mice, rats and rabbits this was done by either force feeding them or by contaminating their food with these segments, though the former method was preferred. The human patients were given the spores either in the form of infected segments or as suspensions of concentrated spores obtained by centrifuging a sieved emulsion of the crushed segments. These were administered by enclosing them in gelatin capsules. In the experiment with the dog the spores were fed by mixing the segments and concentrated



emulsion of spores with its meat. The jackdaws were given a homogenised suspension of spores by injecting it through a fine catheter tube into the gullet.

Mites were fed with the spores and with the eggs of Moniezia by merely introducing the segments, or suspensions of either spores or eggs into the culture tubes .

The spore suspensions and infected segments that were fed in all these experiments were kept for long periods in the refrigerator without their viability or infectivity being affected as shown by their ability to infect Hymenolepis nana of mice .

In examining the experimentally infected worms to see if the infection had taken, several methods were adopted. As a rule the worms were crushed, segment by segment, to see whether spores or developmental stages could be detected. But as this was quite a tedious process, especially when a large number of worms were involved, a more satisfactory method had to be adopted. It was found, quite accidentally, that Newton's Crystal violet stain for chromosomes was retained by the spores even though the other structures had lost the stain on treatment with alcohol. This was found during attempts at staining

sections by this method - a method used by Jones (1943-) in his studies on the cytology of Hymenolepis anthocephalus and Diorchis reynoldsi. It was then found that whole worms stained in this way showed up the spores in a striking manner and that even the presence of a single spore could be detected in this way. The other method of detecting infected worms was by sectioning them, a method that was most reliable when dealing with infections of short duration where only developmental stages were present; and also in mite infections when it was the only way of making sure that the spores seen were actually in the tissues of the mite and not just the spores that were swallowed by the mite, and that were still in the lumen of the mite's intestine.

Culture Methods for Mites - Finally, the experiments with oribatid mites involved methods of extraction of the mites from turf samples and methods of rearing them for various periods of time after infecting them with eggs or spores. The mites were recovered from leaves or turf samples, from Kent and St.Albans. The mites received from Oxford were already extracted by a colleague Mr. C.H.Fernando who was kind enough to do this for me. The samples from Kent were kindly brought by Mr. D. Winter of the Department of Entomology.

The mites were extracted by means of a modification of Rayski's (1945) method, which was based on the same principle though on a smaller scale.- A 9-inch cube biscuit tin was used for this purpose. A ridge 1/2 inch wide was soldered along the inner wall of this tin at about 2 inches from the bottom. On this was placed a coarse wire-mesh. The lid of the tin was fitted with a bulb-holder into which a 40 watt bulb was fixed projecting inwards. The samples of turf or the collections of leaves were now placed on the wire-mesh, with the grass surface downwards. The bulb was lit with the lid closed, and the apparatus left for 6-8 hours. The mites then fell off on to a piece of filter paper made in the shape of a cone and placed under the wire-mesh. This was necessary as otherwise the water that condensed down the sides of the tin tended to collect at the bottom and drown the mites. The condensation of water was minimised by making 2 or 3 holes about the diameter of a pencil on the lid of the tin. The mites collected after extraction in this way were sorted out with the aid of a fine camel-hair brush and later placed in appropriate culture tubes or examined for evidence of natural infection with either microsporidia or other protozoa, or with cysticercoids of tapeworms.



The tubes used for keeping the mites for long or short periods were made on the same plan as suggested by Rayski (1945) . Each tube was 3 inches in height and 1 inch in diameter, and was fitted with a cork through which a 1/2 inch hole had been bored. To the inner side of this hole in the cork was pasted on a circular piece of very fine-mesh copper netting by means of some 'Eostik'. Each culture tube was then prepared by adding 1 c.c of distilled water to it followed by plugging the bottom with a wad of cotton wool. After the water had soaked into the cotton wool, the food material for the mites was added. This consisted of dried poultry manure and sphagnum moss, both of which had been previously sterilized in an autoclave. This was a necessary step to reduce the growth of fungus in the culture tubes. Some of the tubes were prepared by a method used by Mr. D. Hinton of the Department of Entomology, using a mixture of plaster of paris and powdered charcoal instead of the cotton wool. This was done in the following manner. - 9 parts of plaster of paris were mixed with 1 part of activated charcoal powder and then made into a thick paste with distilled water. A small quantity of this paste was then added into a culture tube , and by tapping the tube several times



at the bottom, the paste was made to settle at the bottom of the tube to about a height of about 1-1 1/2 inches. Distilled water was then added till the paste, which by now had hardened, was saturated with the water. As the water evaporated after a few days the colour of this mixture turned a pale grey and then it was time to add a fresh amount of water.

All culture tubes were placed in a deep trough which was covered with an air-tight lid. The walls and lid of this trough were covered with black paper and into the trough was added water to keep the humidity within it at a high level as this was a special requirement for the mites. The tubes were placed on a stand in the trough so that no water could get into them.

The mites were added into these culture tubes by means of a fine camel hair brush. The cotton wool and the plaster/charcoal mixture were kept moist by adding a few drops of distilled water every few days. All experiments on the mites were carried out at room temperature.

#### General account of the infection in Moniezia

It was found that Nosema helminthorum occurred in both species of Moniezia found in sheep in this

country, namely M. benedeni and M. expansa. The table below summarises the findings on 181 Moniezias studied in detail, although several more were examined which were not considered in working out the incidence of infection. Each worm examined was given a number from M-1 to M-181. and the table shows details of only the infected ones.

Worm No.	Scolex Region	Anterior Segments	Posterior Segments	Remarks
M 1.	-	-	+	Only detected In sections.
M 5.	+	++	++	
M 20.	++	++	++	
M 27.	+	++	++	
M 28.	- ve	- ve	++	
M 29.	- ve	+	++	
* M 35.	- ve	++	++	
* M 40.	+	++	++	
M 55.	- ve	++	+	
M 59.	- ve	+	++	
M 60.	- ve	- ve	++	
M 63.	- ve	- ve	+	
M 66.	- ve	- ve	+	

\* Moniezia benedeni .

Worm No.	Scolex Region	Anterior Segments	Posterior Segments	Remarks
M 85.	- ve	- ve	++	
M 93.	++	+	-ve	Only scolex and anterior 2 $\frac{1}{2}$ inch.+
M 98.	- ve	- ve	++	Only last 3 inch.+
M 110.	- ve	- ve	+	Only last 6 inch.+
M 112.	- ve	++	+++	
M 115.	- ve	- ve	+	
M 119.	+	++	-	Posterior segments missing.
M 125.	- ve	- ve	+	
M 126.	++	++	++	Patchy opaque white areas seen naked eye
M 128.	- ve	- ve	+	
M 135.	++	++	++	Same as M 126.
M 136.	- ve	+	++	
M 139.	- ve	+	+	
M 142.	- ve	+	++	
M 148.	- ve	- ve	+	
M 154.	++	++	-	Posterior segments missing.
M 166.	++	++	++	
M 177.	- ve	+	+	
M 179.	+	- ve	++	
M 180.	-	-	++	Gravid segments from 2 lambs from
M 181.	-	-	++	Aberystwyth.



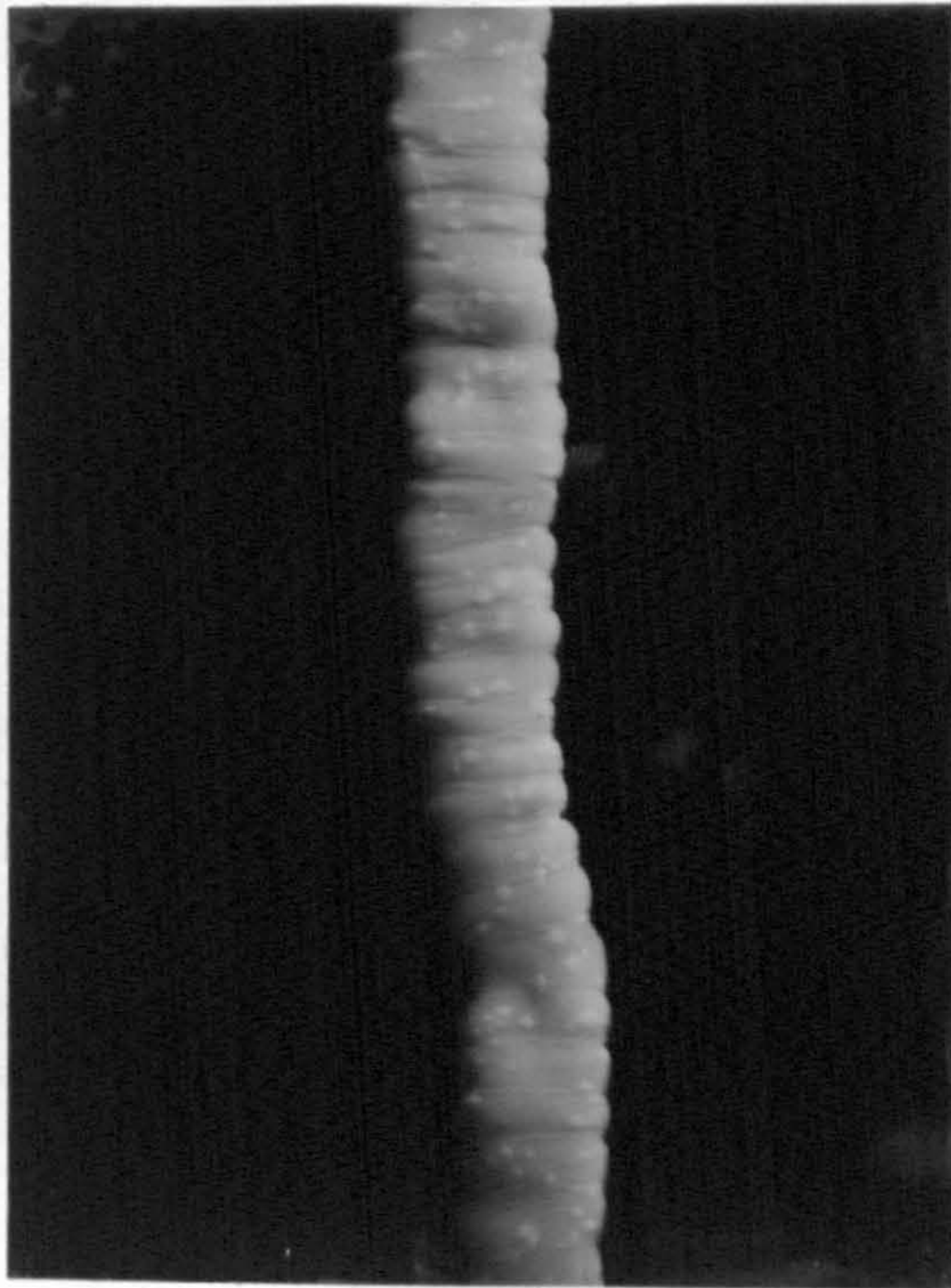


Fig. A.

Photograph of Moniezia expansa showing  
white opaque areas of infection  $\cdot(\times 14)$ .



From this table it is seen that 34 of the 181 *Moniezias* studied in detail were infected with the microsporidian, i.e. about 18 % of worms show this infection. It is, however, possible that among the others labelled as uninfected a few may have shown only developmental stages which were therefore missed by the method of examining smears of crushed segments. Such infections could only be detected by careful sectioning of all regions of these worms, which is not practicable. As far as possible only worms complete with scolices were counted in this table for working out the incidence of infection, as otherwise there was a possibility of portions of broken off segments of the same worm being more than once counted as separate worms. Of the worms that were clearly identified it was found that 14 were *Moniezia expansa* and 2 *M. benedeni*.

As the table indicates only in a few worms was the infection easily detected with the naked eye due to the presence of patchy opaque white areas of infection ( Fig. A ), an observation that was made by Weiser(1951) for his *Nosema bischoffi* in *Moniezia benedeni*. The majority of worms, however, showed no detectable difference from the normal worms either in appearance

or behaviour. For instance they showed the same degree of movement as the normal worms and continued to react to heat for the same length of time as the normals since the time of collection.

In all worms that showed infection the posterior segments invariably showed infection though the scolex and more anterior segments were free. Only one worm N 93, showed infection in the scolex and anterior segments while the posterior gravid segments were clean. One worm showed infection in the scolex and neck regions, no infection in the intermediate segments, and again a moderately heavy infection in the posterior segments (N 179).



### Chapter III

#### Morphology and Stages in the development of Nosema helminthorum

##### Introduction

In his original account of this parasite Koniez (1879) gave the following information, " J 'ai trouvé à plusieurs reprises dans le Taenia expansa une Psorospermie très-voisine de ces corpuscles qui déterminent chez le Ver à soie une maladie trop connue, la pébrine. Ces Protistes étaient répandus en quantité prodigieuse par tous les tissus de leur hôte. J 'ai pu étudier leur reproduction. Un Taenia denticulata m'a offert une seule fois la même Psorospermie, mais il vivait de compagnie avec un Taenia expansa bourré de ces parasites ". Subsequently in 1887 he named it Nosema helminthorum and gave some further details, " les spores du parasite s'observent en énorme quantité dans les mailles des tissus; elles pénètrent à l'interieur des ovules dont elles n'empêchent pas toujours l'évolution, et c' est ainsi qu'elles passent à de nouveaux hotes; ces spores sont ovales et mesurent près de 5  $\mu$ . sur 2 $\mu$  ,5 ..... elles se reproduisent par scissiparité et j'ai fréquemment ,

récontre les masses sporigènes ".

Moniez gave the name N. helminthorum as he believed it to be a common parasite of many helminths among which he mentioned the nematodes Toxocara cati. Although Moniez obviously saw most of the stages of this microsporidian, he has unfortunately not given any illustrations and his descriptions were not detailed. There is little doubt, as I have pointed out earlier, that Labbé(1899) was referring to the same organism which he reported from the same tapeworms, but his reason for separating it into a new genus, Plistophora, seems inadequate since what he probably saw were groups of spores within a common membrane due to the destruction of host tissues around. This appearance is quite often seen in sections and even in fresh smears ( see later), and there is no evidence at all of any pansporoblasts. Weiser's (1951) description of the microsporidian which he found in a single specimen of Moniezia benedeni from a sheep is more complete in that he has described and drawn a few of the developmental stages as well as the spores. His reason for placing this parasite in a new species Nosema bischoffi, however, is unjustified since he bases the separation on the size



of the spores, which he claims are larger than those of N.helminthorum . I have earlier given sufficient evidence for considering that he was dealing with the same parasite. Weiser was unfortunately handicapped by the fact that he had limited material to work on and so his description was lacking in certain details.

It would appear from the above that the past accounts of Nosema helminthorum have been both incomplete and inadequate and it is therefore necessary to give a detailed account of all the stages met with in the present work

#### Early Stages and Schizonts

Immediately the sporoplasm enters the host tissues it multiplies by binary fission or by multiple fission. Both these processes have been referred to as schizogony. It was originally thought that there was only one such phase of schizogony, but of recent years<sup>at</sup> there has been a tendency to define two phases of this process. The significance of these two phases will be discussed later but it may be mentioned here that the second is generally a preparation for spore formation , and several writers have compared this stage to gametogony in other Sporozoa, seeing at this stage either a sexual differentiation or a process of



autogamy. Unfortunately this latter phase in the life cycle of microsporidia is so rarely met with, as Naville(1931) points out, that not many observers believe in the existence of such a phase. In the present work I have been fortunate in being able to see and study this phase thoroughly in two of the infected worms, and the appearances strongly suggest the presence of an autogamy in the second phase of development of the parasite. This description will therefore be given under the following headings - primary schizogony, and secondary schizogony (autogamy).

Primary Schizogony - Although a large number of infected worms were studied, developmental stages were only met with in a few of them. This was because in the others the development of the parasite had already gone to the spore stages, and this condition generally obtained throughout the length of the worm. In a few worms, however, the various stages of development were met with and so a complete picture of the cycle of development was obtained.

The sporoplasm of the microsporidia of arthropods escapes from the spore in the gut of the host and has then to find its way to the particular tissue which it attacks. This wandering stage has been called the 'planont'

by Stempel (1909). He believed that the planont stage multiplies actively by binary fission and moves around by amoeboid movements in the alimentary canal and in the inter-cellular spaces of the host body. After leading such an extra-cellular life in the haemocoel these planonts enter host cells and then begin intra-cellular development. Kudo (1944) points out that though no workers have actually seen actively multiplying planont stages as described by Stempel, no one doubts the existence of an extra-cellular stage between the emerged sporoplasm and the intra-cellular schizonts.

In the microsporidia of tapeworms, however, the question arises whether there could be such an extra-cellular stage at all. The emerged sporoplasms, as will be shown later, are brought directly in contact with the host cuticle whence they work their way into the parenchyma of the worm passing between the sub-cuticular cells. Once they reach this position they start on the first phase of schizogony. This stage during which the parasite passes through the cuticle and between the sub-cuticular cells can be compared with the planont stage, but there can be little doubt that there is no multiplication during this phase.

The earliest stage of Nosema helminthorum

Explanation of Plate II

Developmental stages of Nosema helminthorum from  
smears stained in Giemsa stain.

2-3. Early schizonts with compact nuclei.

4-5. " " with nuclei composed of several  
granules.

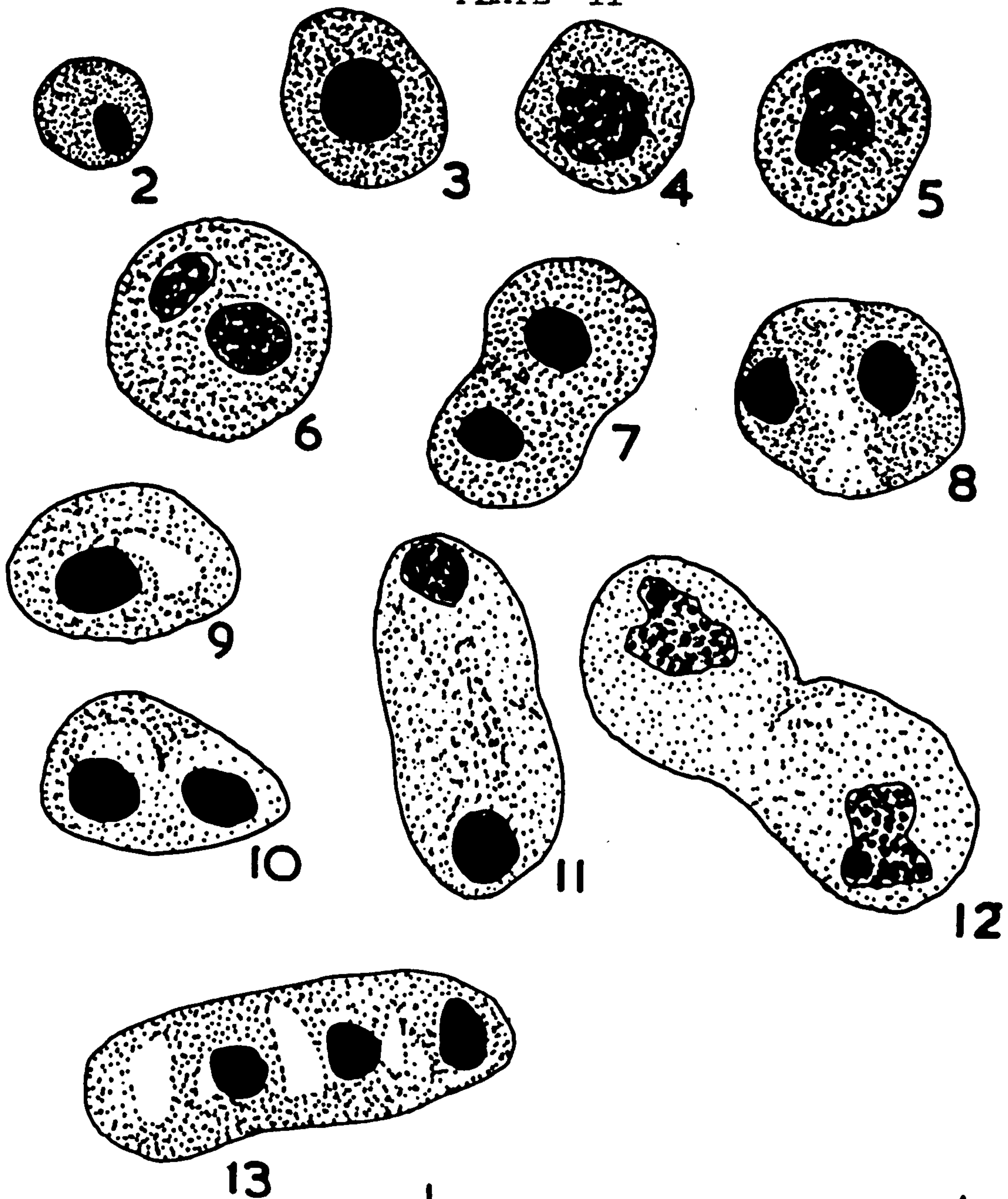
6-8. Early binuclear schizonts.

9-12. Later binuclear schizonts with fainter cytoplasm  
uninuclear schizont  
and one (9) showing clear area round nucleus.

13. An elongate tri-nucleate schizont.



PLATE II



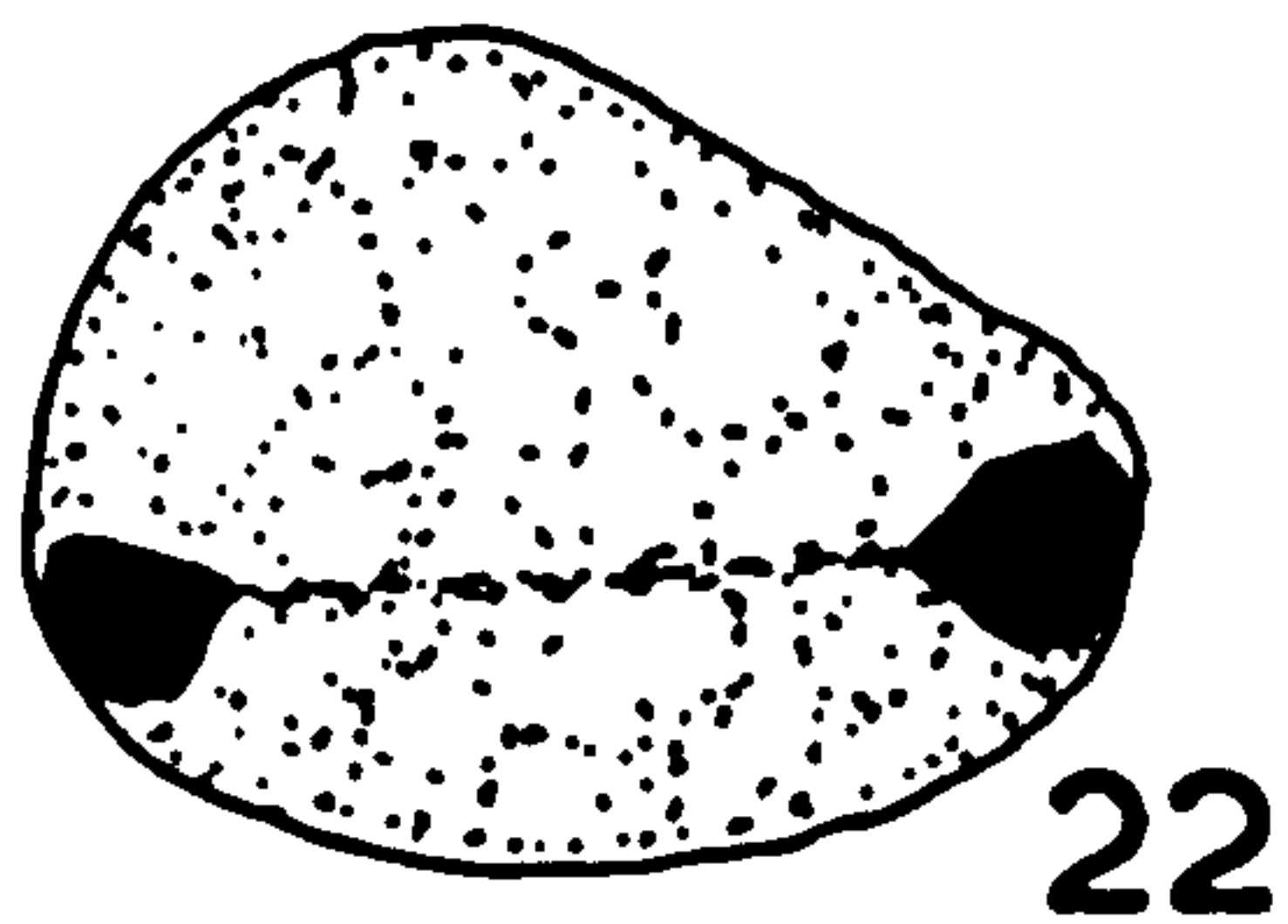
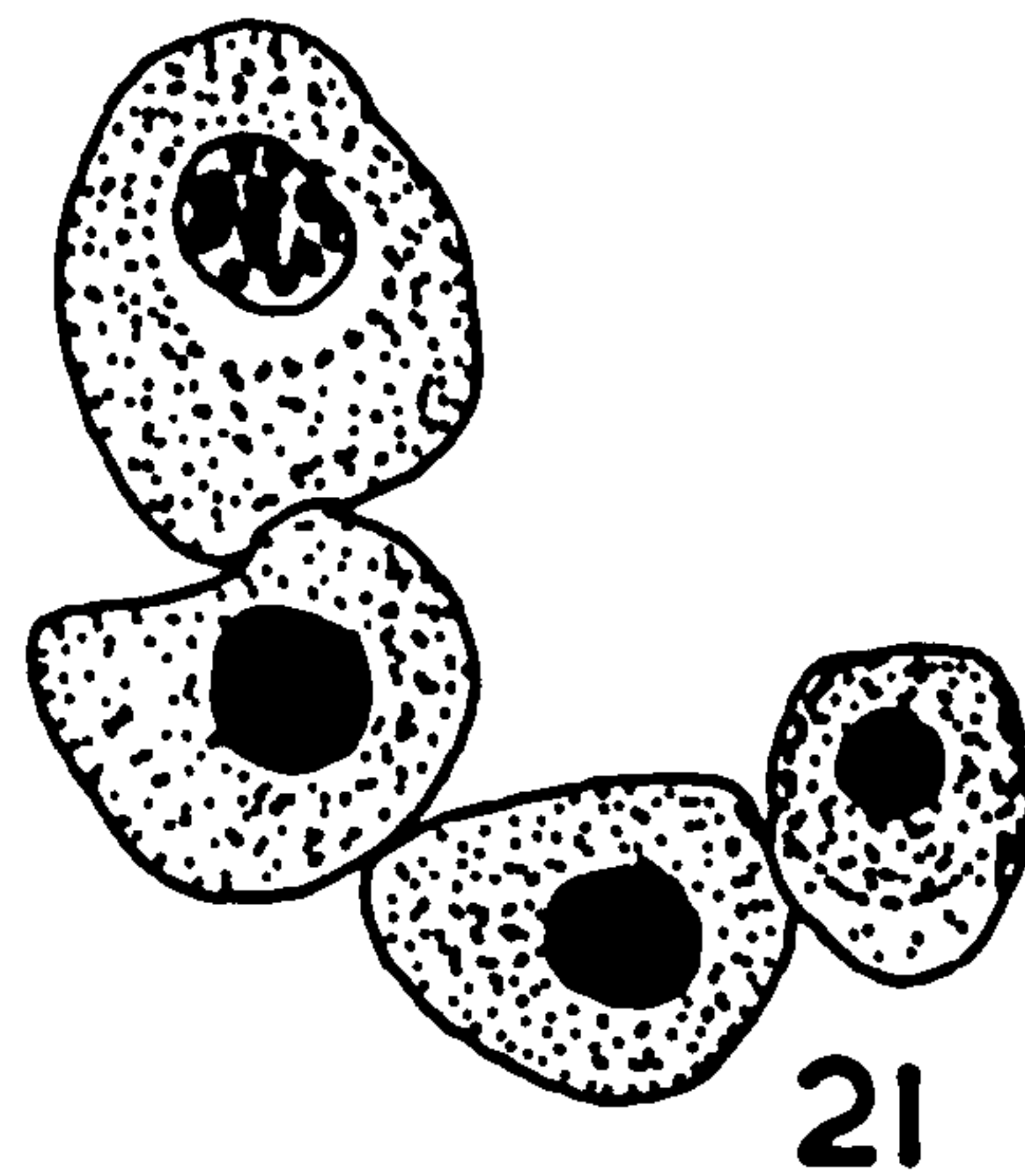
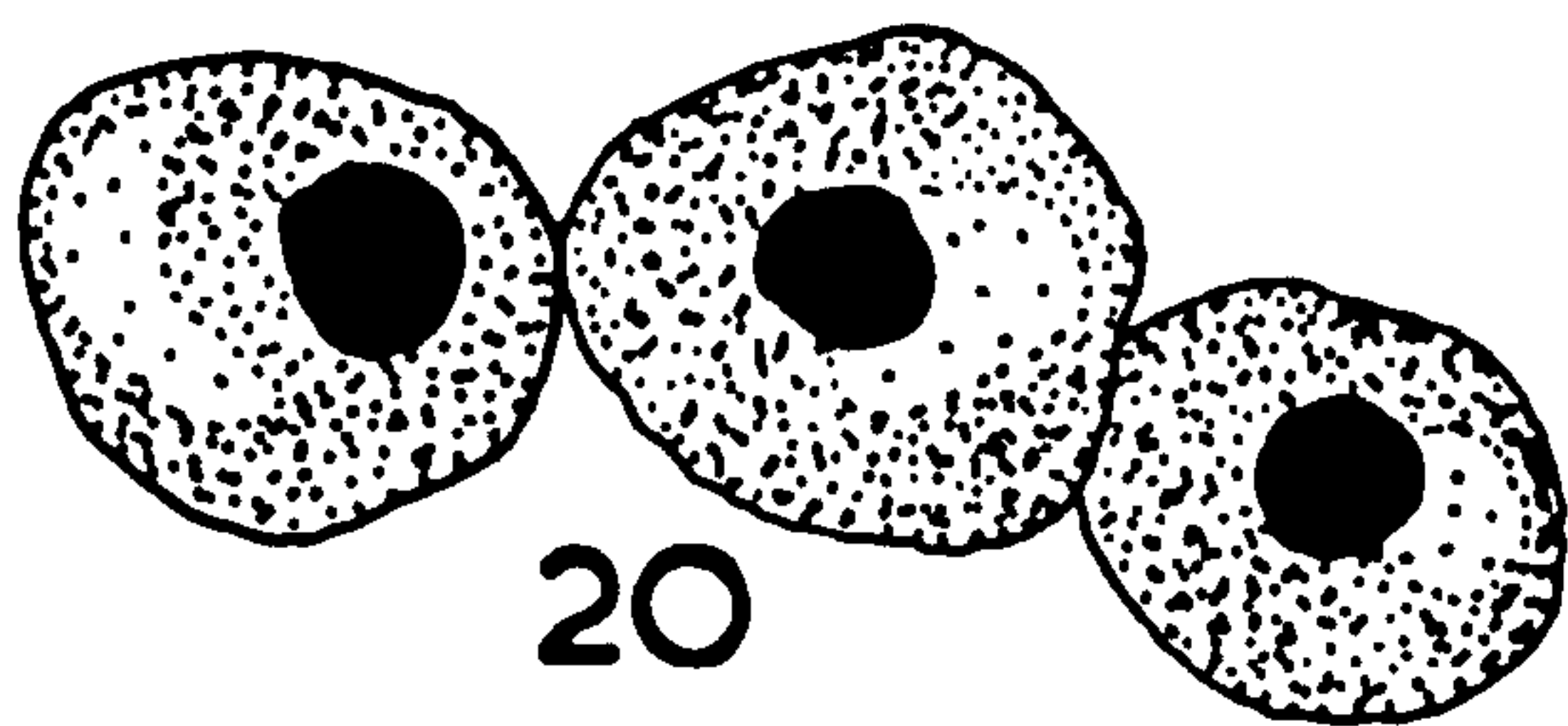
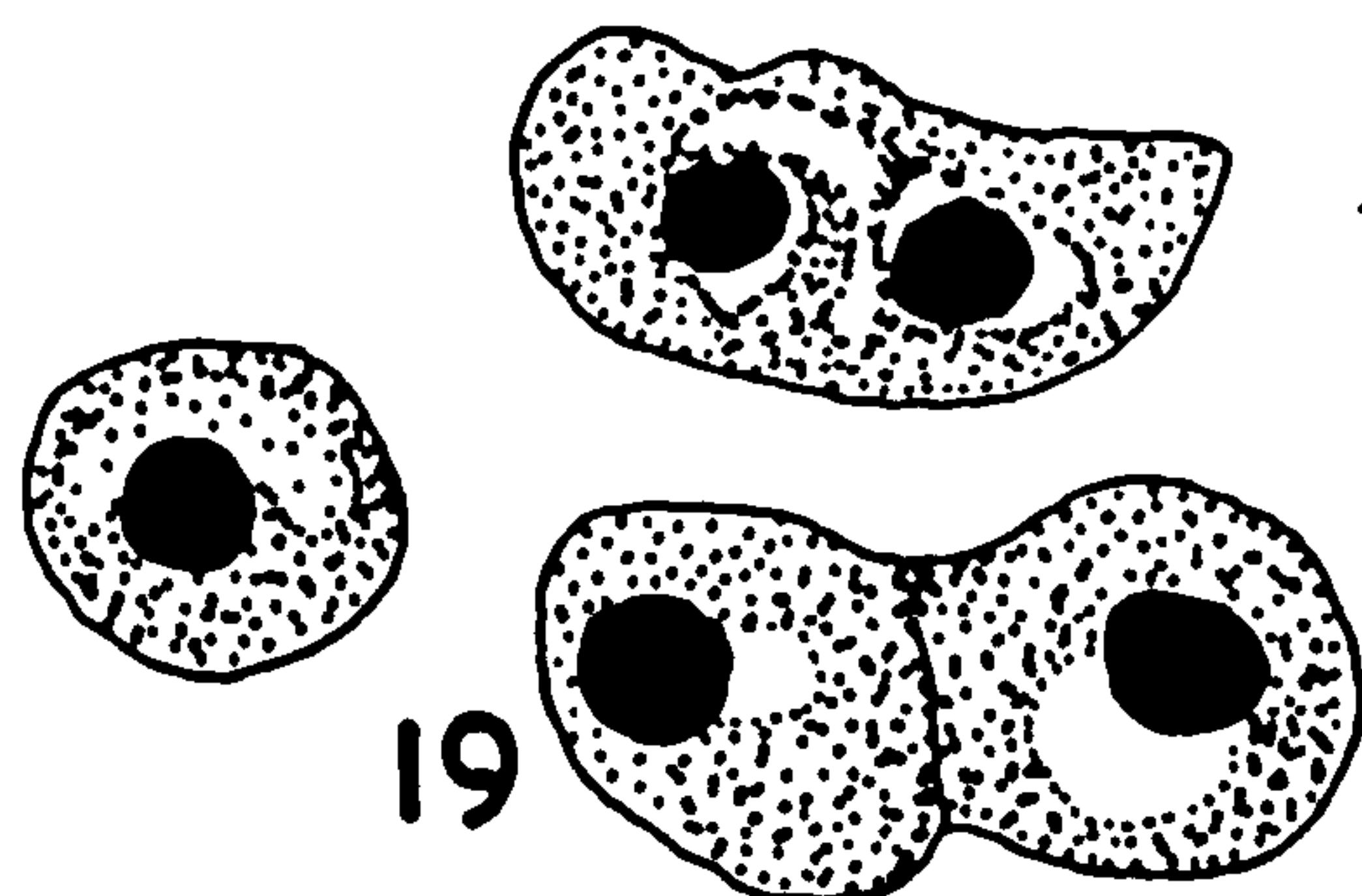
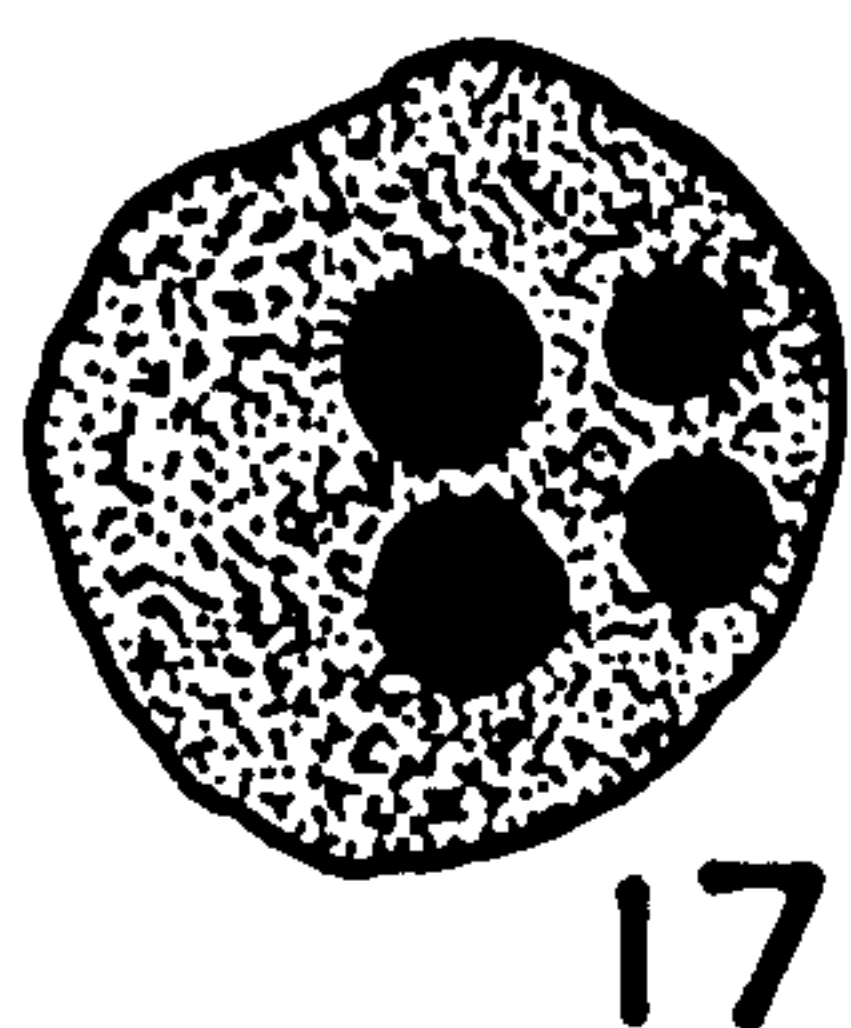
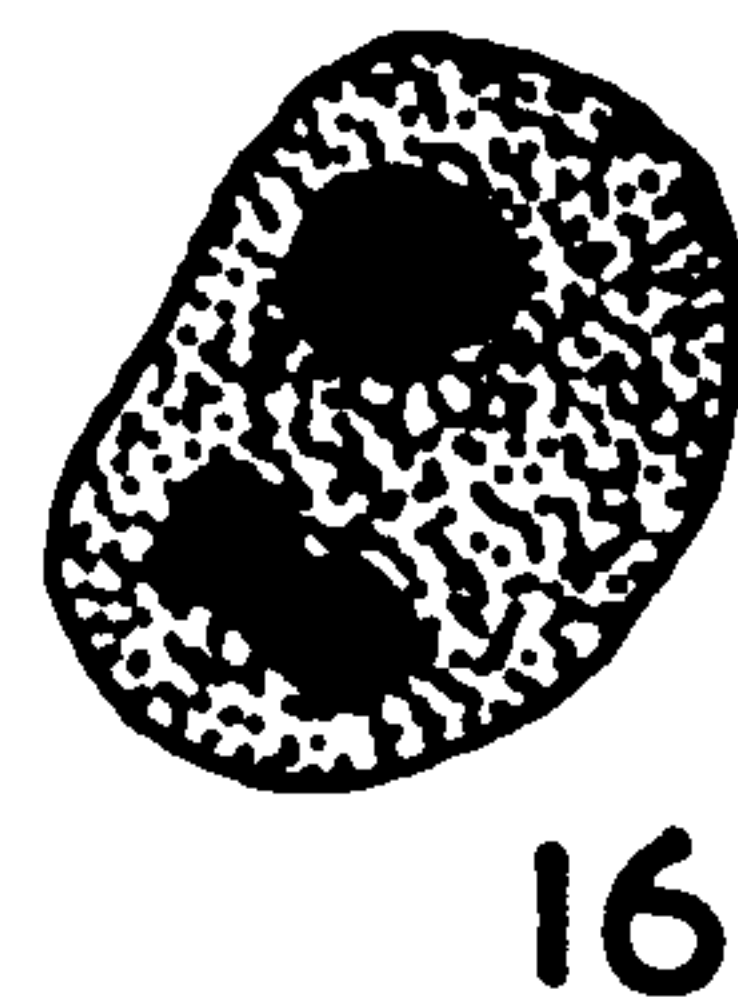
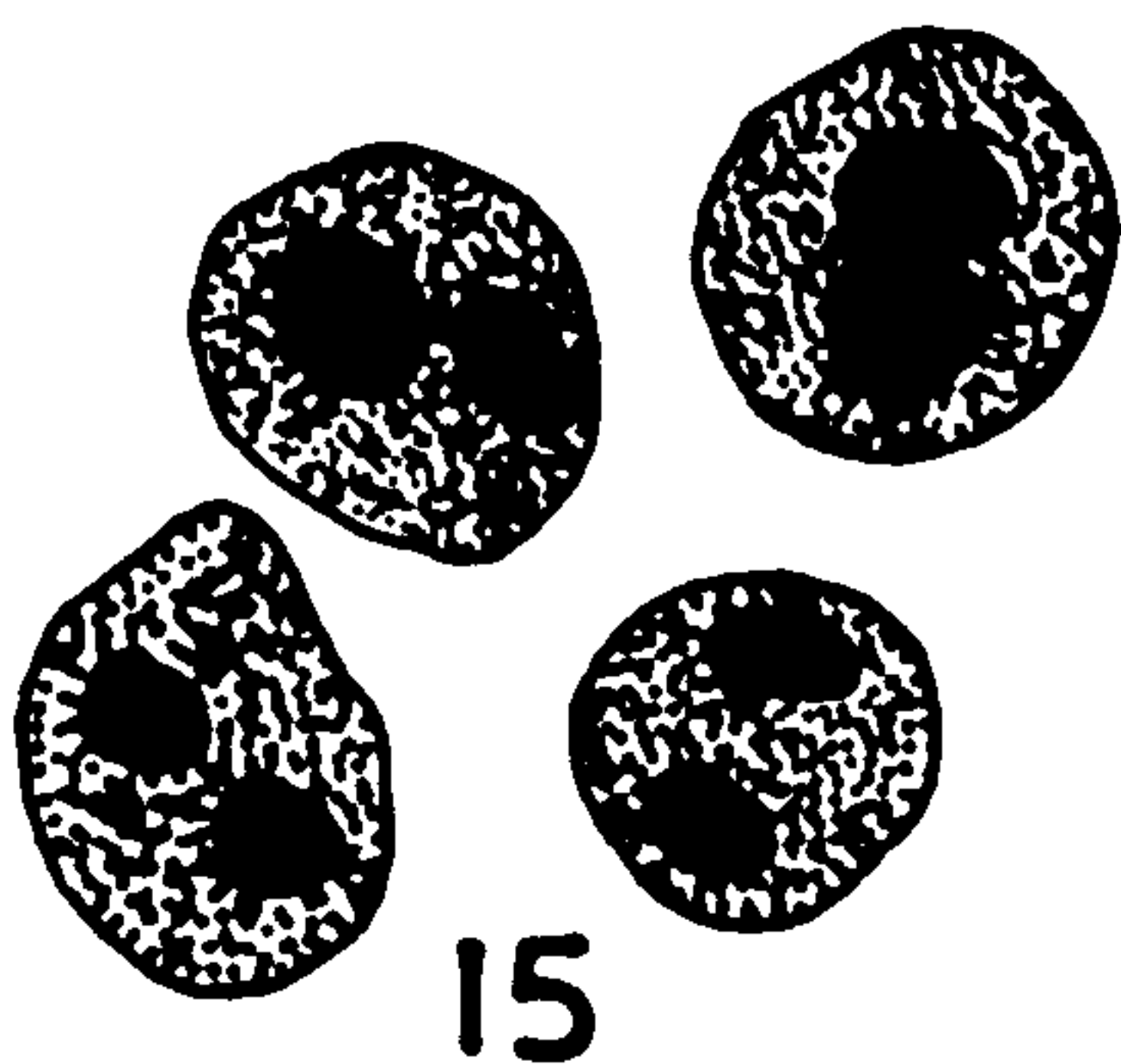
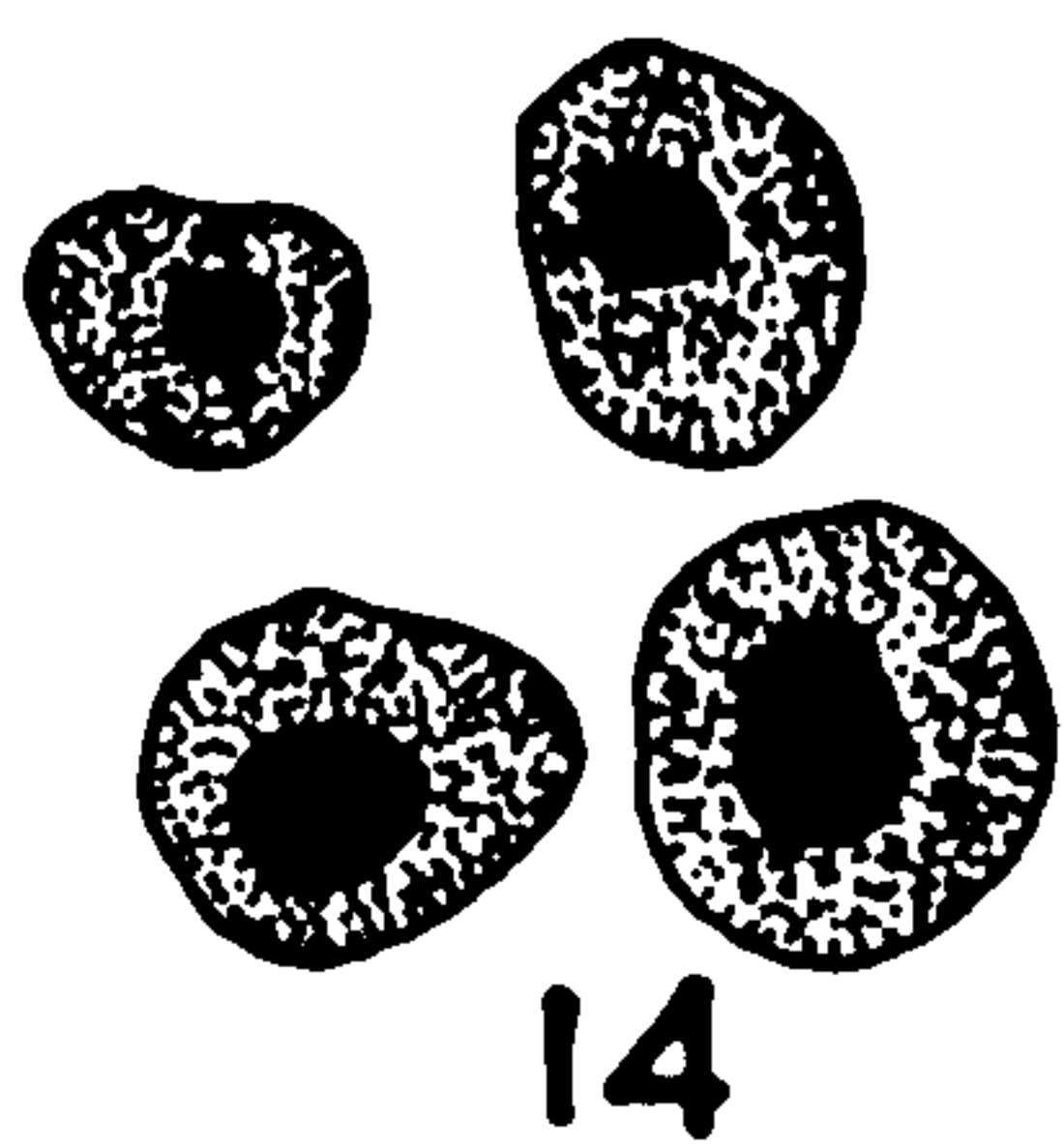
10  $\mu$ .

### Explanation of Plate III

Developmental stages from sections stained by  
Giemsa-Colophonium method.

- 14. Early uninuclear schizonts.
- 15. Early binuclear schizonts.
- 16. Tri-nuclear schizont.
- 17. Tetra-nuclear schizont.
- 18. Early schizont showing dividing nucleus  
showing dumb-bell shape.
- 19. Later schizonts with clear zones around nuclei
- 20-21. Schizonts in chains.
- 22. Later binuclear schizont with connection between  
nuclei.

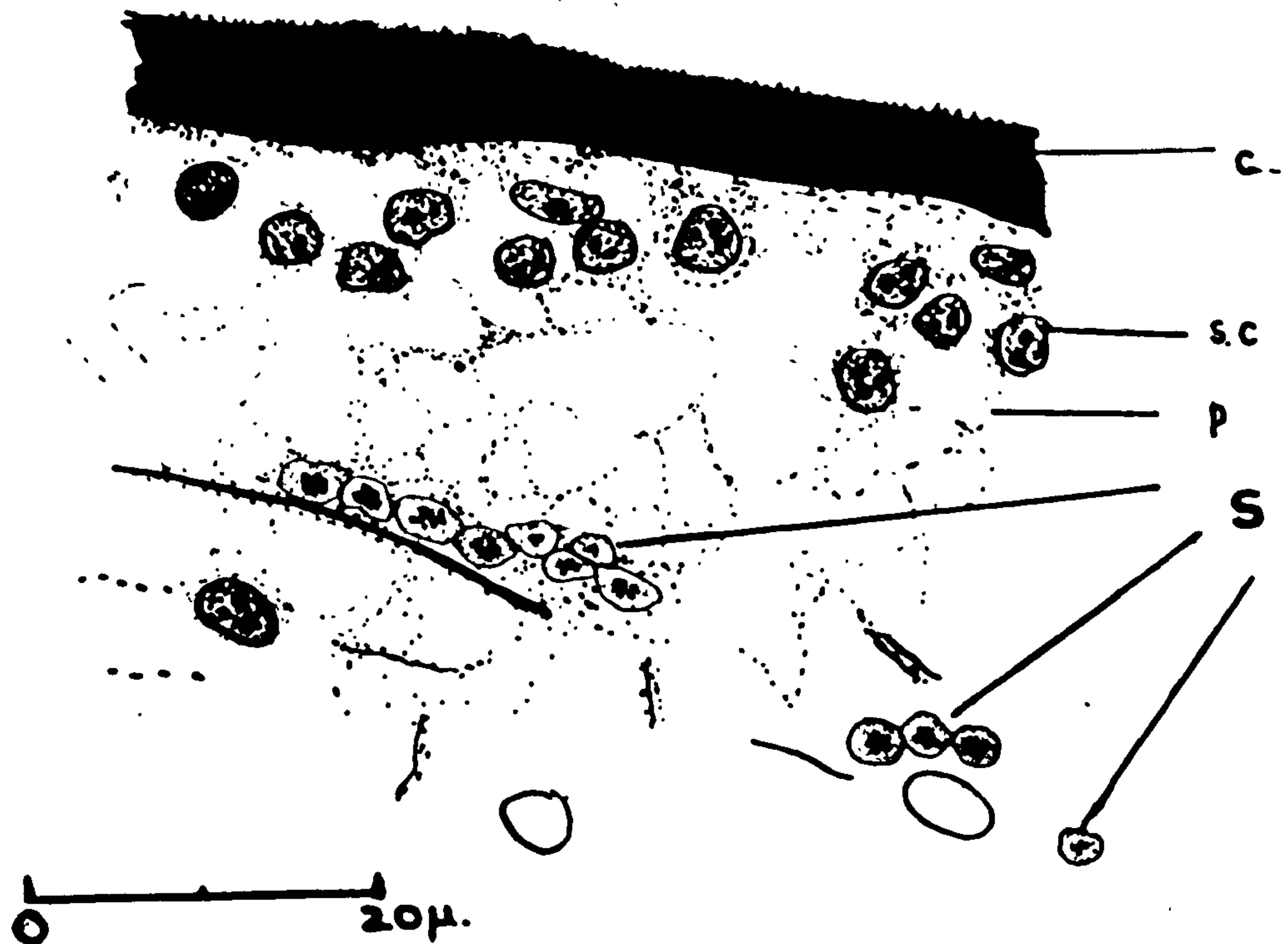
PLATE III



10 $\mu$ .



# PLATE IV



21 a.

Section of M. expansa showing  
schizonts in cortical region.  
Giemsa- Colophonium.

- c - Cuticle
- s.c. - Nuclei of sub-cuticular cells
- p - Parenchyma
- S - Schizonts in chains

is seen in sections ( Plate III , Fig. 14) as a small spherical body of 1.4  $\mu$ . diameter, having a very deeply staining cytoplasm. These stages are found generally quite close to the sub-cuticular cells. The nucleus in these forms is a dense red granule at the centre. The cytoplasm stains so deeply that in order to make out the nuclear details the sections that have been stained by the Giemsa-Colophonium method have to be markedly over-differentiated. They now increase in size and soon the nucleus divides producing a bi-nucleate body (Fig. 8) (Plate III , Fig. 15). Most of these bi-nucleate schizonts divide into two by simple binary fission, an appearance that is quite easily seen in smear preparations ( Plate II , Fig. 7, 11, 12 ). Although actual details of the nuclear division are not often observed it is clear from Fig. 18 (Plate III), that the process is one of amitosis. The nucleus elongates and divides into two and a nuclear bridge remains between the two for some time. Appearances such as these have been seen by several workers in other microsporidia, for instance Stempell (1909) in Nosema bombycis , Fantham and Porter (1912, 1914) in N. apia and N. bombi , Kudo (1921b) for N. baetis and recently Canning (1953) for N. locustae.



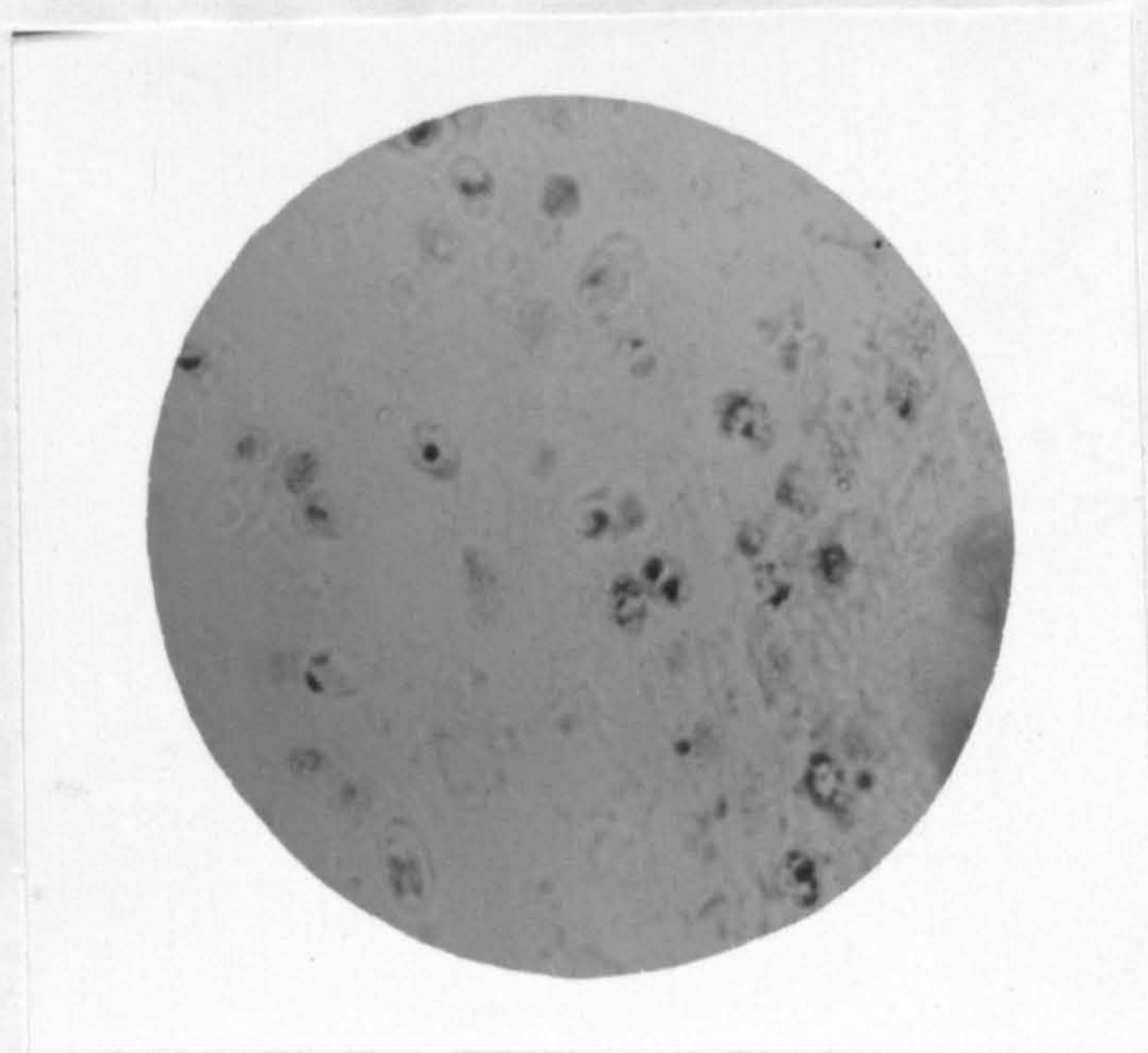


Fig. B.

Uninuclear and bi-nuclear schizonts (I ary)  
from section of M.expansa. ( X 1200 ).  
( Giemsa-Collophonium )



As schizogony proceeds in this manner the cytoplasm begins to stain less and less deeply and by now there appears a clear zone around the nucleus ( Plate II, Fig 9,10 Plate III, Fig. 19 ). Some of the schizonts do not divide by binary fission but remain spherical and undivided while the nuclei continue to divide, and in this way schizonts with 2,3, or even 4 nuclei are formed which give rise, no doubt, to daughter schizonts by cytoplasmic cleavage ( Plate III, Fig. 16, 17). Occasionally chains of schizonts are seen ( Plate III, Fig. 20, 21, Plate IV, Fig 21 a) which are most probably formed by a series of binary fissions, but it is quite possible that such an appearance may result from cytoplasmic cleavage of an elongate multinucleate schizont ( Plate II, Fig. 13) , as was shown by Weiser (1951).

In some of the schizonts that stain less intensely the nuclei appear less compact and seem to be made up of a number of chromatin granules or threads which occupy a greater space than the compact nuclei. These nuclei ( Plate IV, Fig. 21 a) bear a strong resemblance to those illustrated by Kudo (1925 ) for Thelohania legeri. The significance of this appearance of the nuclei is uncertain but it seems possible that the schizonts in



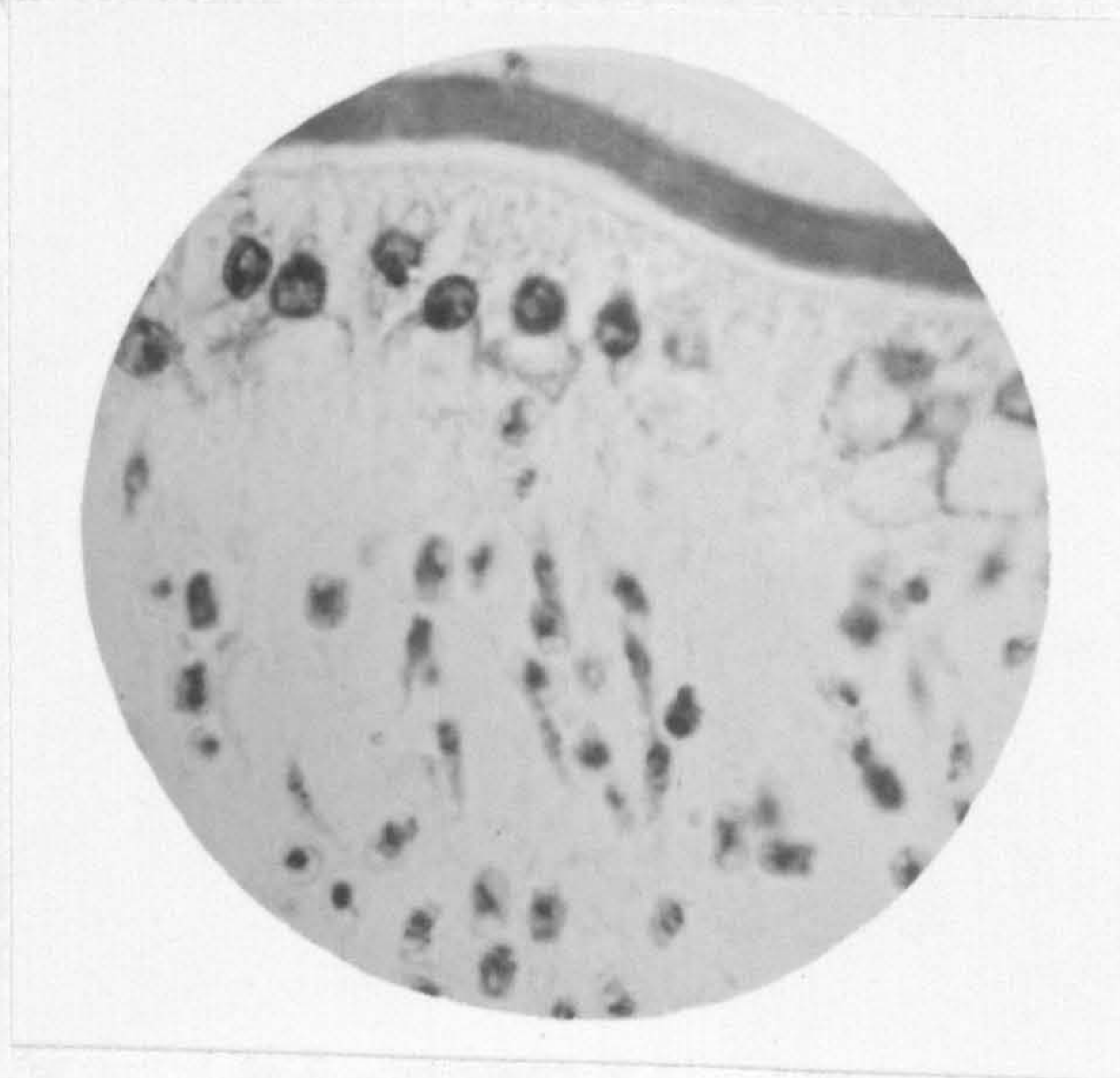


Fig. C.

Uninuclear fusiform schizonts from section of  
Moniezia expansa (M 135) Giemsa-Colophonium  
 ( X 1000 . )

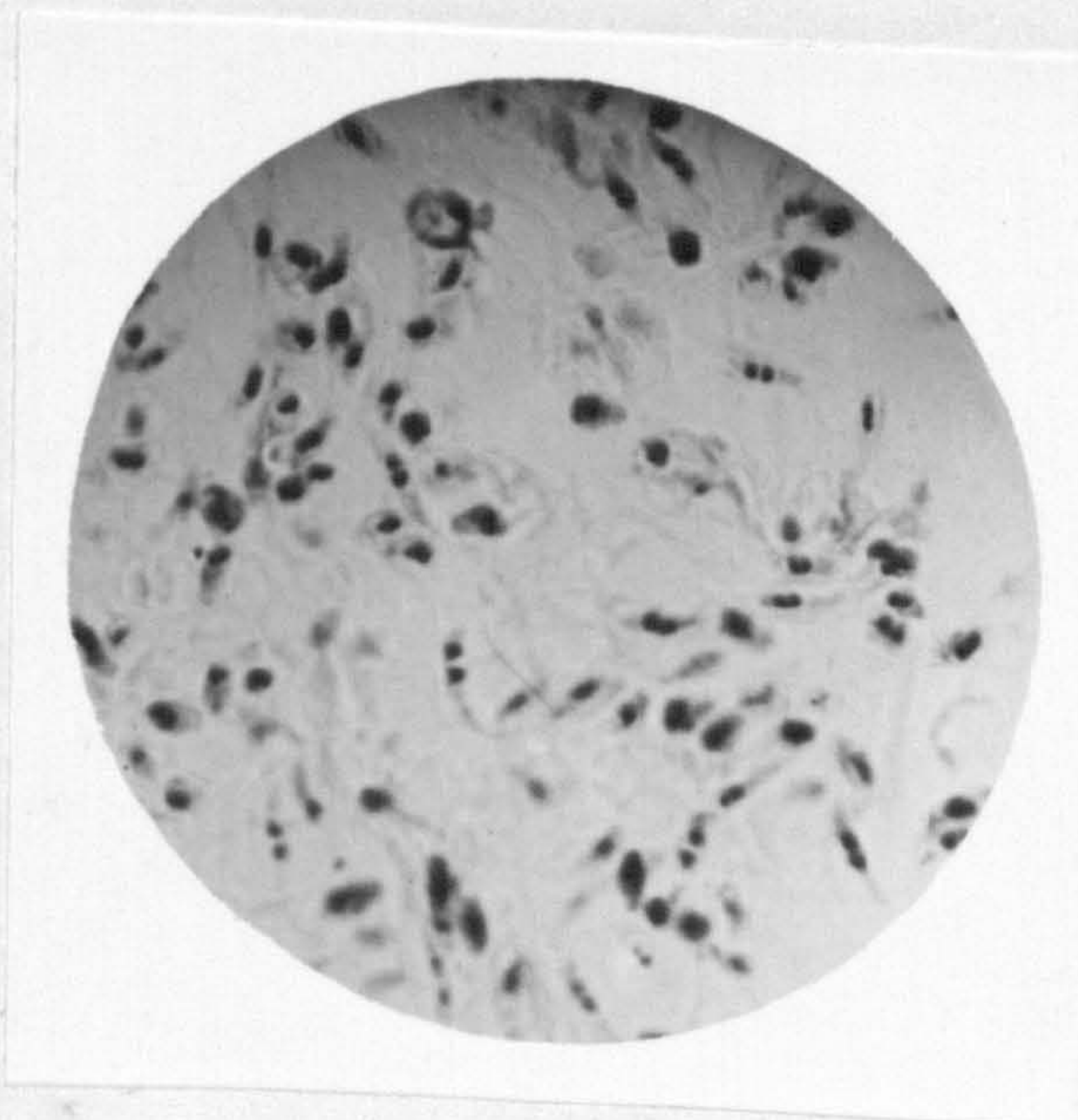


Fig. D.

Bi-nuclear fusiform schizonts from section of  
M. expansa (M. 125) Giemsa-Colophonium ( X 1000 )



which they occur are the precursors of the autogamous schizonts which soon follow.

The above stages are seen in all worms in which developmental stages are met with and in these instances sporonts and spores are relatively scanty.

Secondary Schizogony (Autogamy) - In two infected worms ( M 125 and M 135 ) a peculiar type of cell was observed in large numbers. In both these, the cells were fusiform with extremely long drawn out processes. ( Fig. C, D ). In M 135 the nuclei of these cells were vesicular with one or two large chromatin granules in them ( Fig. C ). These cells bore a strong resemblance to fibroblasts, and many tests and stains were carried out to verify this. Several attempts were made to see if tapeworms kept 'in vitro' after injecting India ink under the cuticle, would show a fibroblastic reaction but with negative results. Sections of M 135 were then stained in Heidenhain's Iron Haematoxylin, Mallory's Triple, Mallory's Acid Fuchsin, Mallory's Phospho-tungstic haematoxylin, van Gieson's and Bielschowsky's Silver stains. All that they revealed was that the processes of these fusiform cells were much longer than they actually appeared in Giemsa stained sections.



Explanation of Plate V.

Sporont stages from smears stained in Giemsa stain.

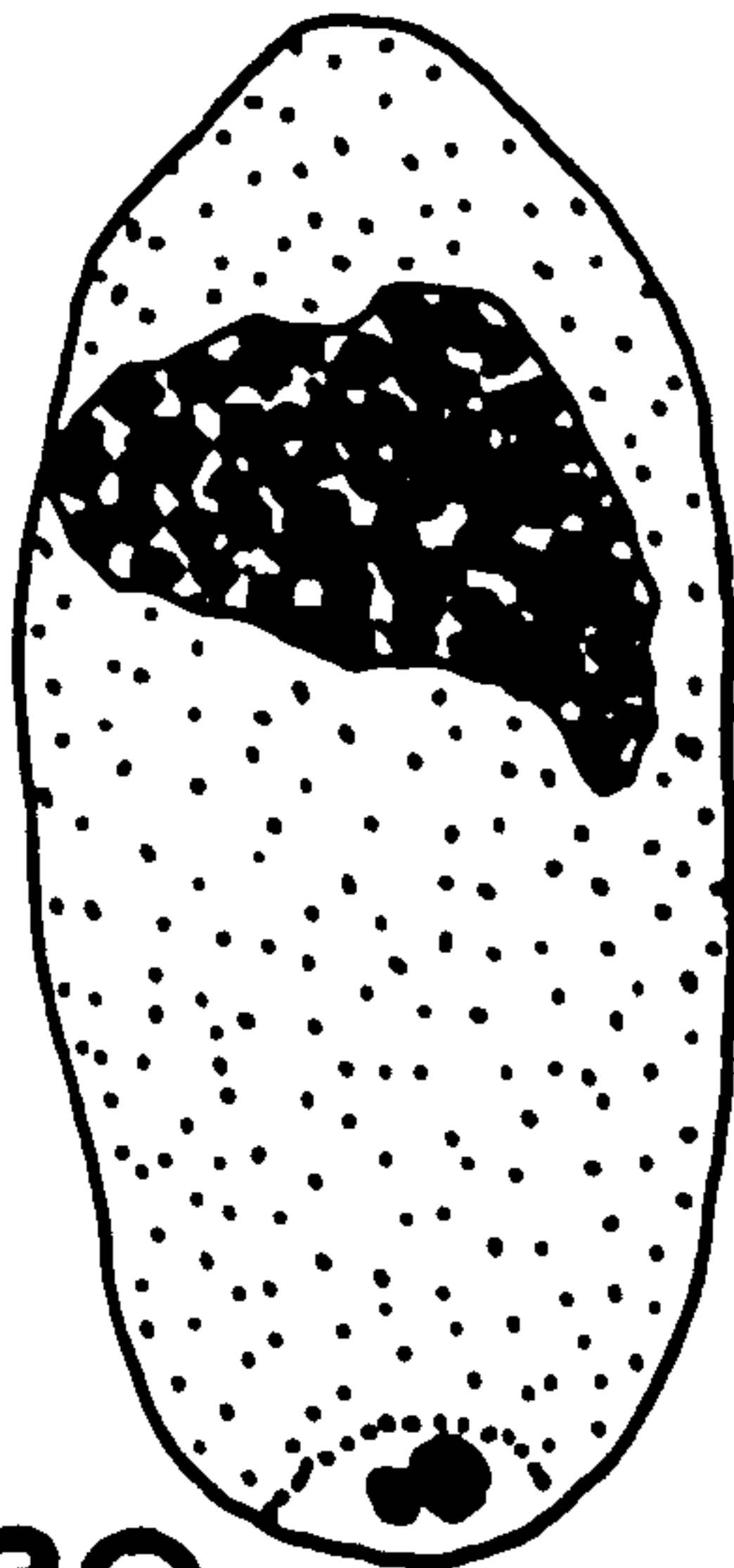
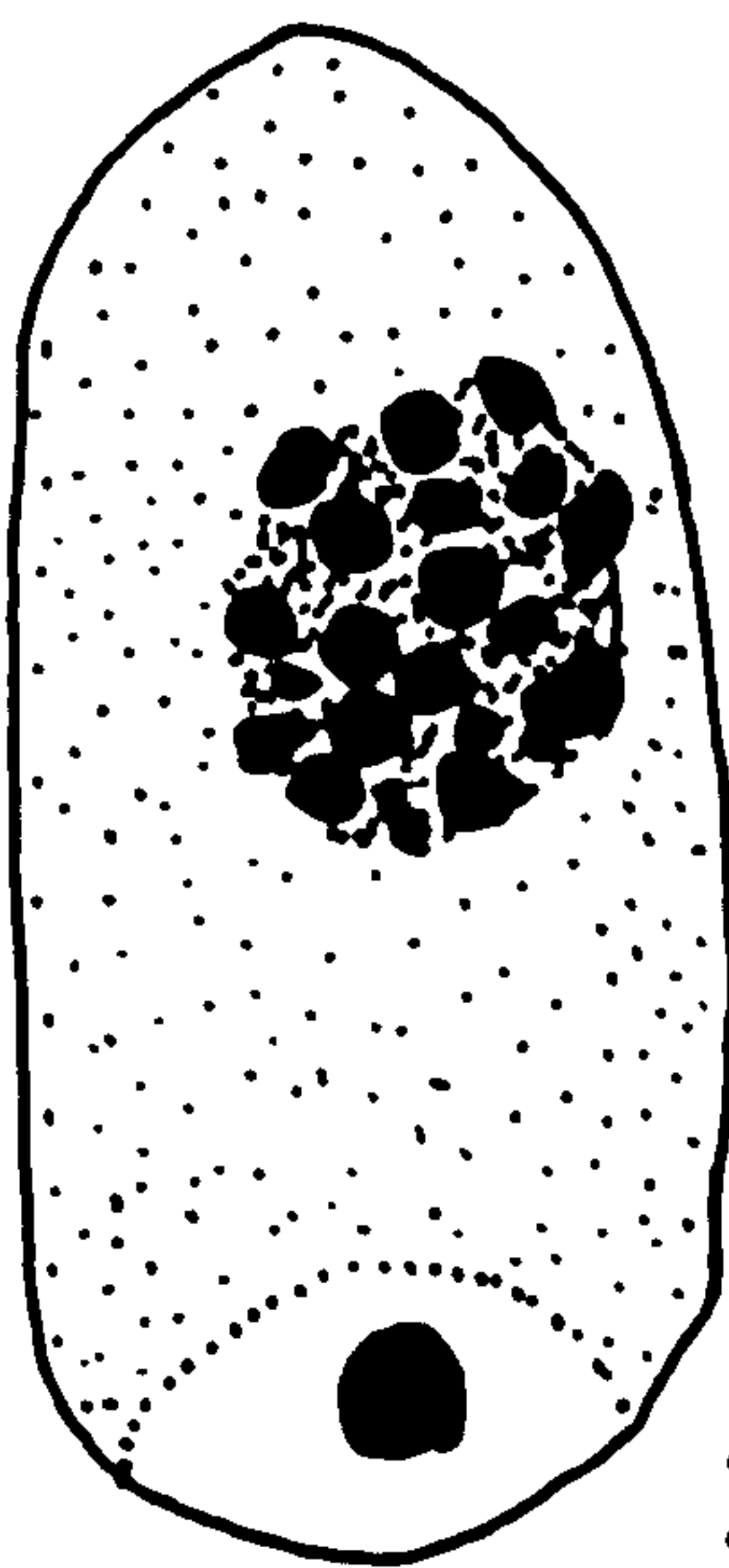
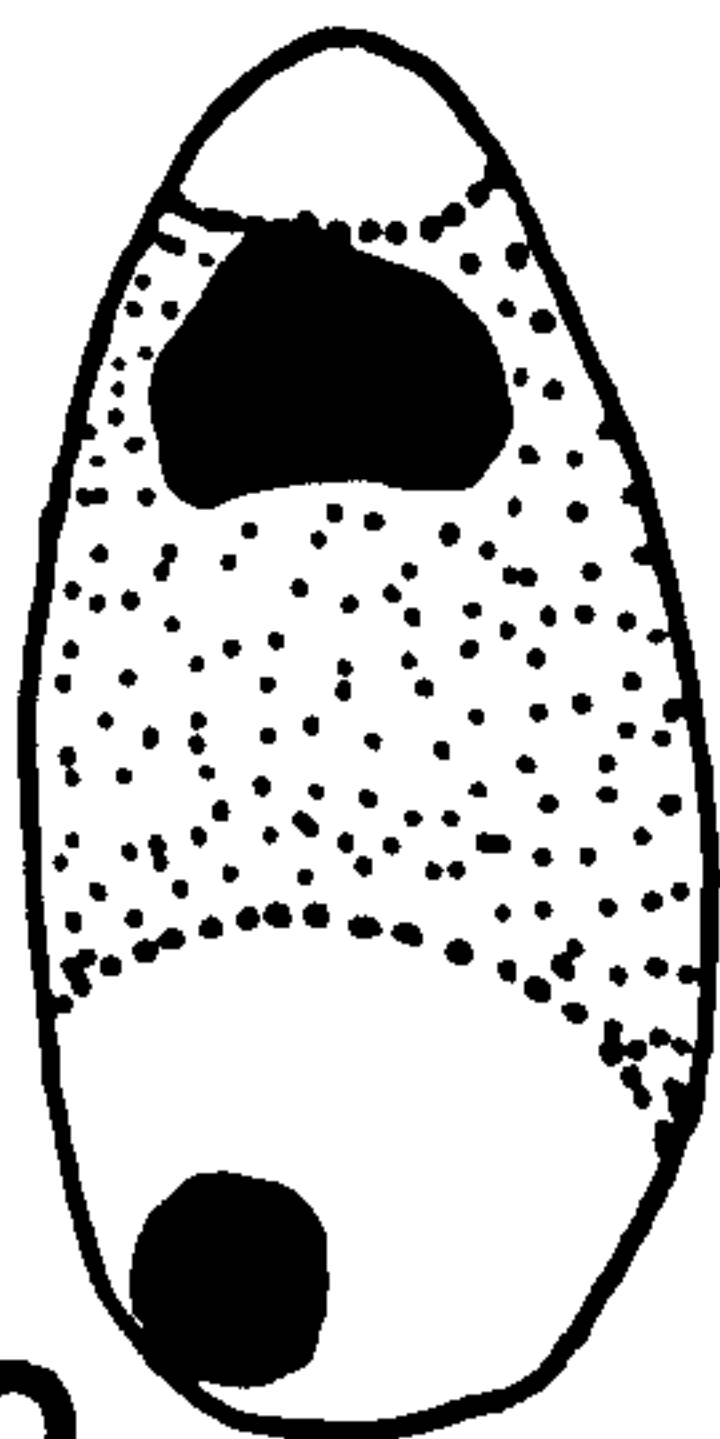
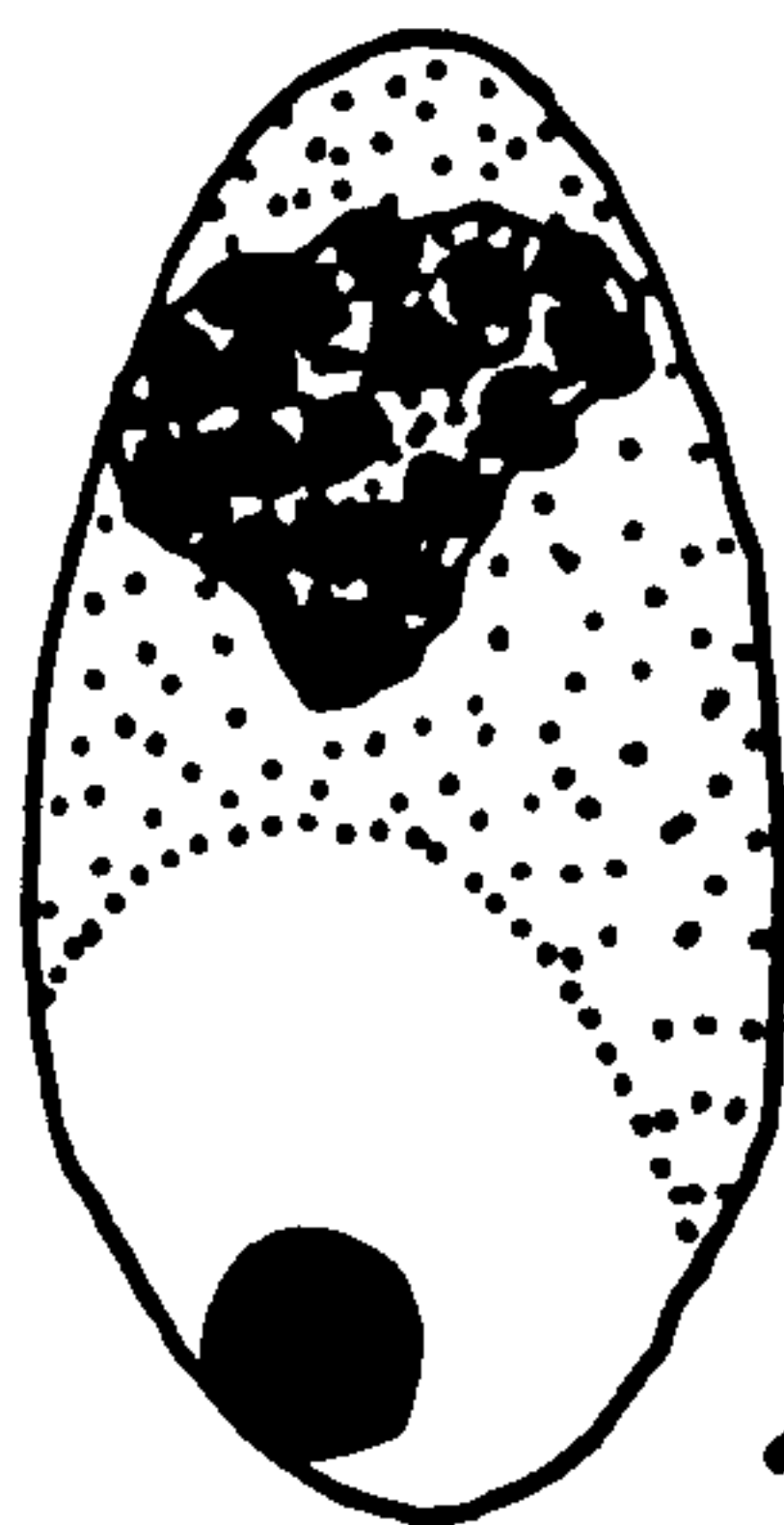
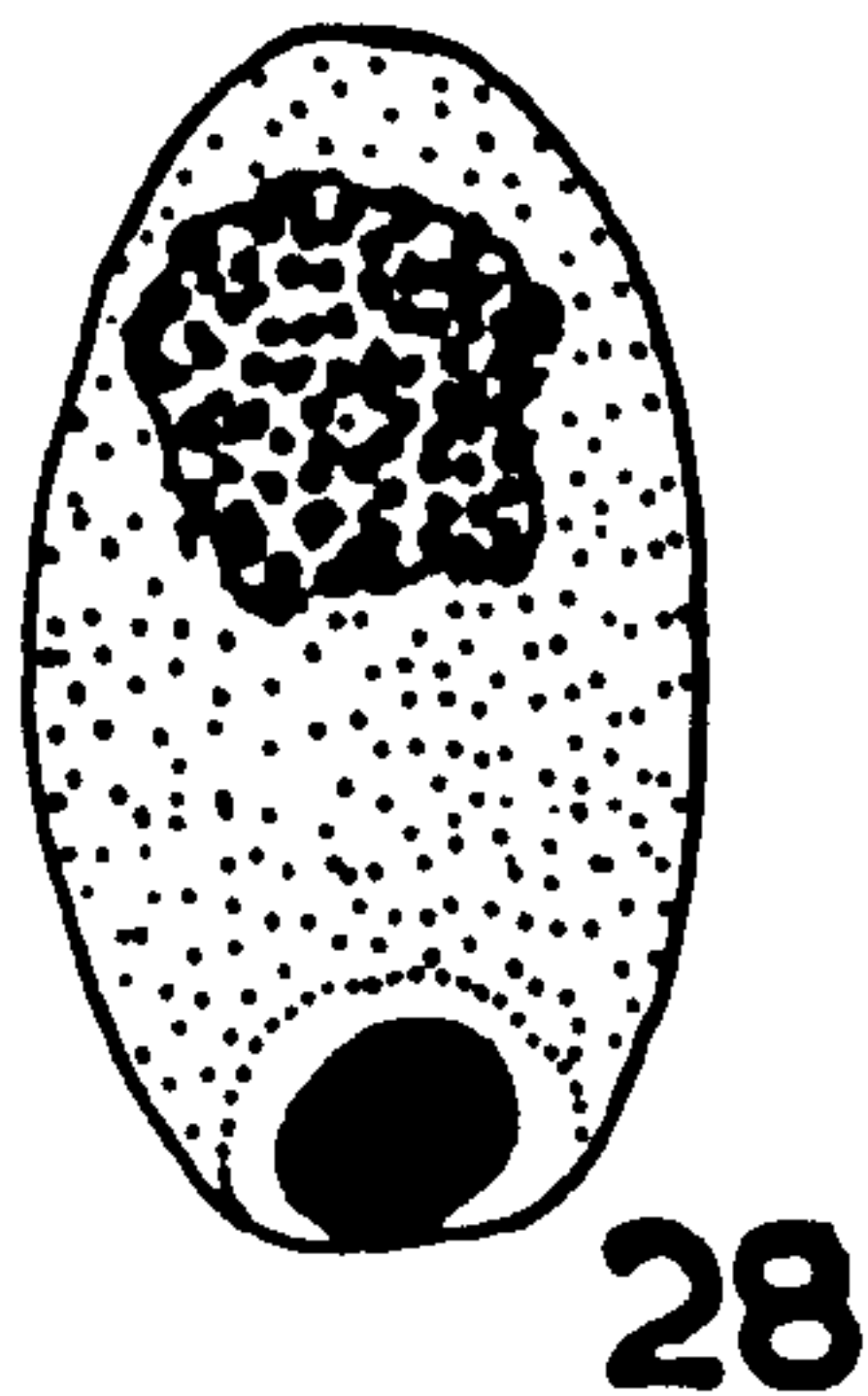
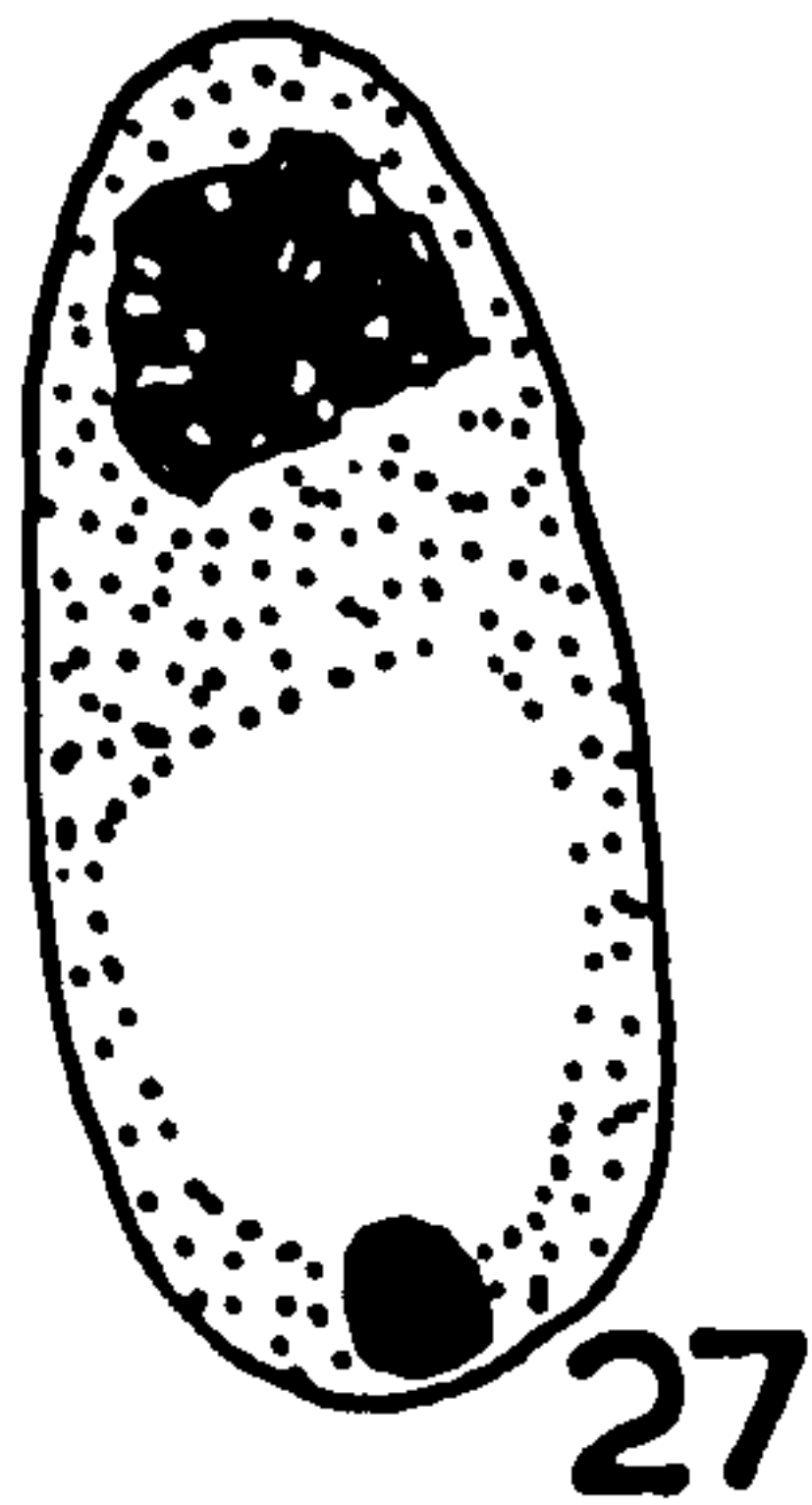
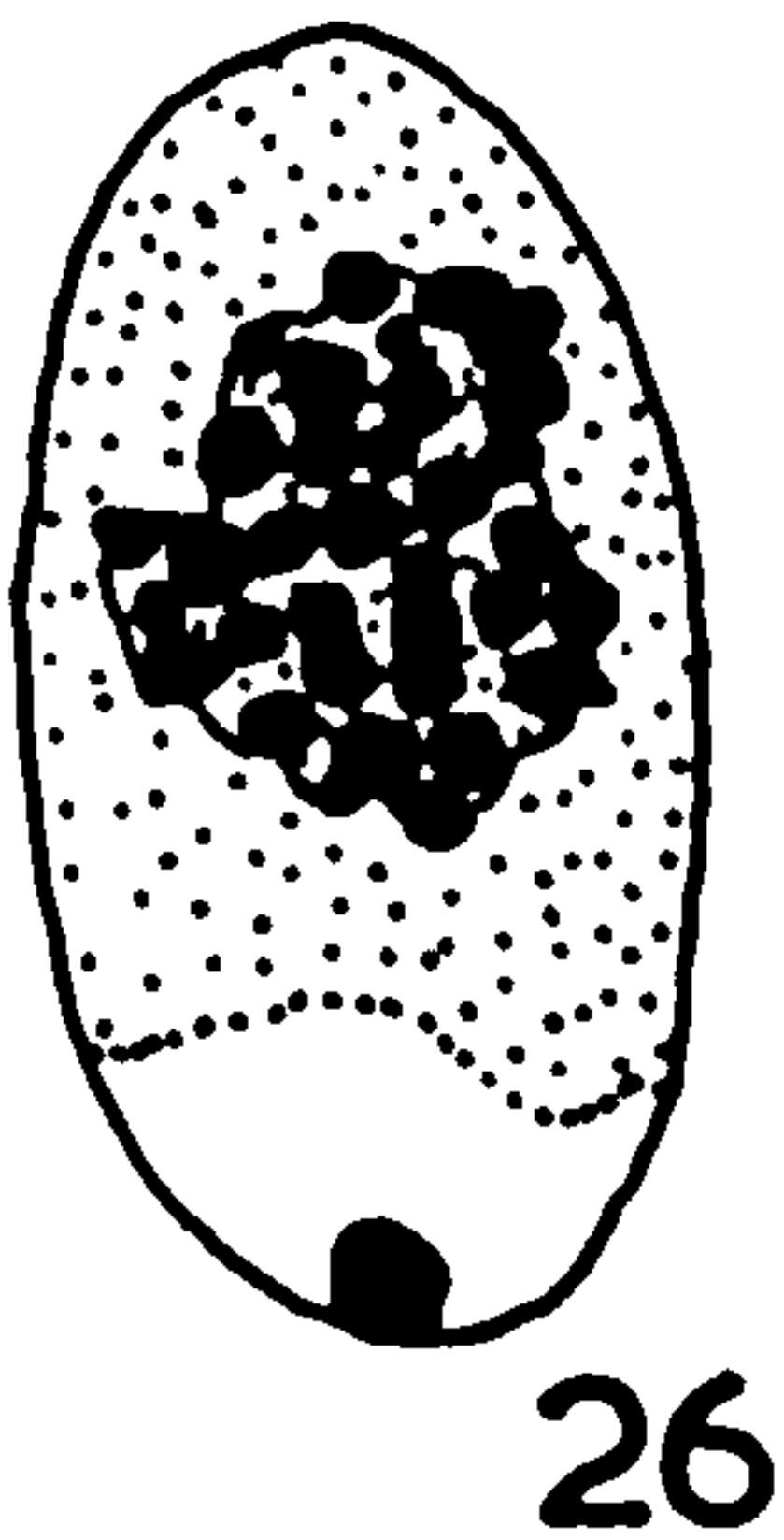
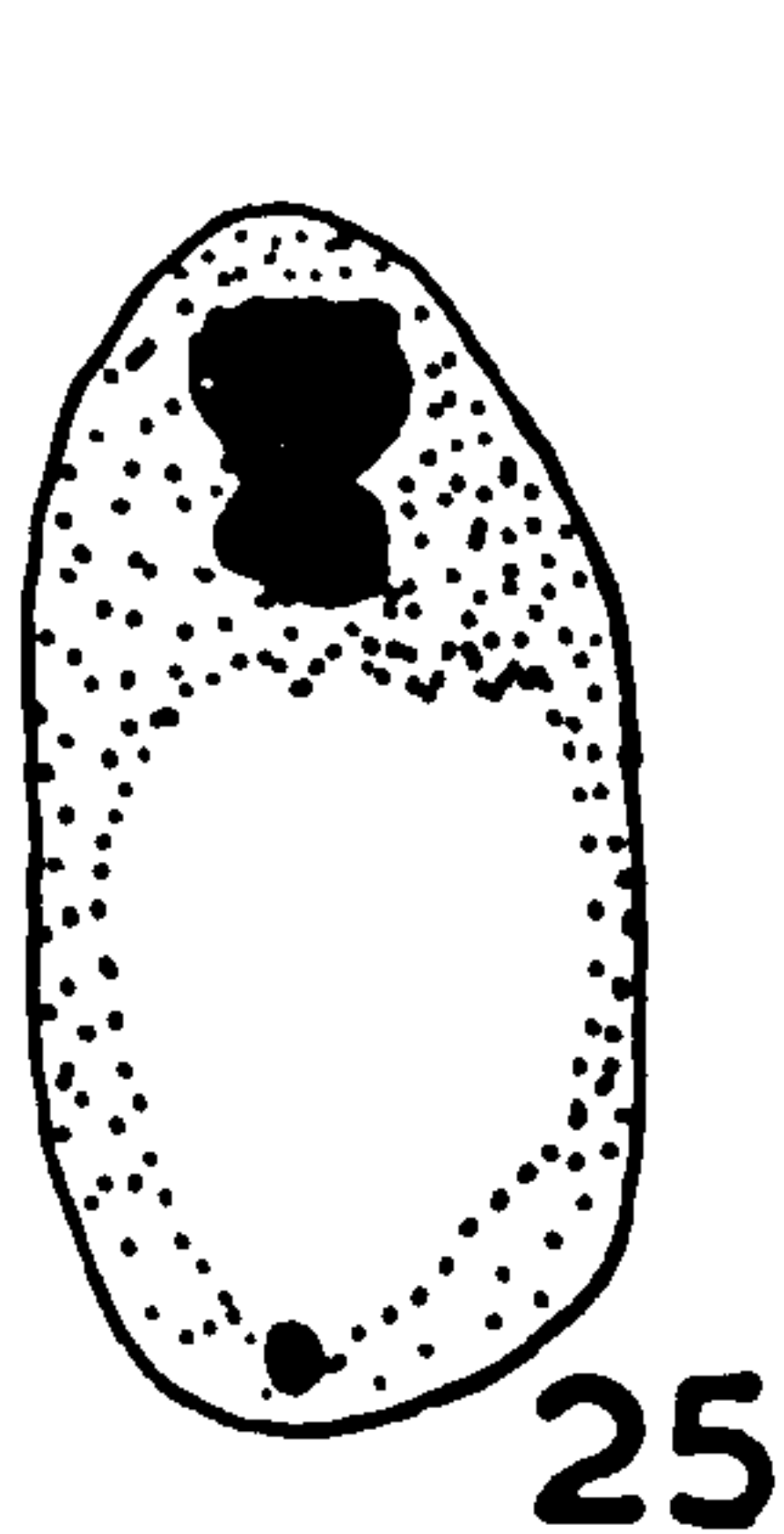
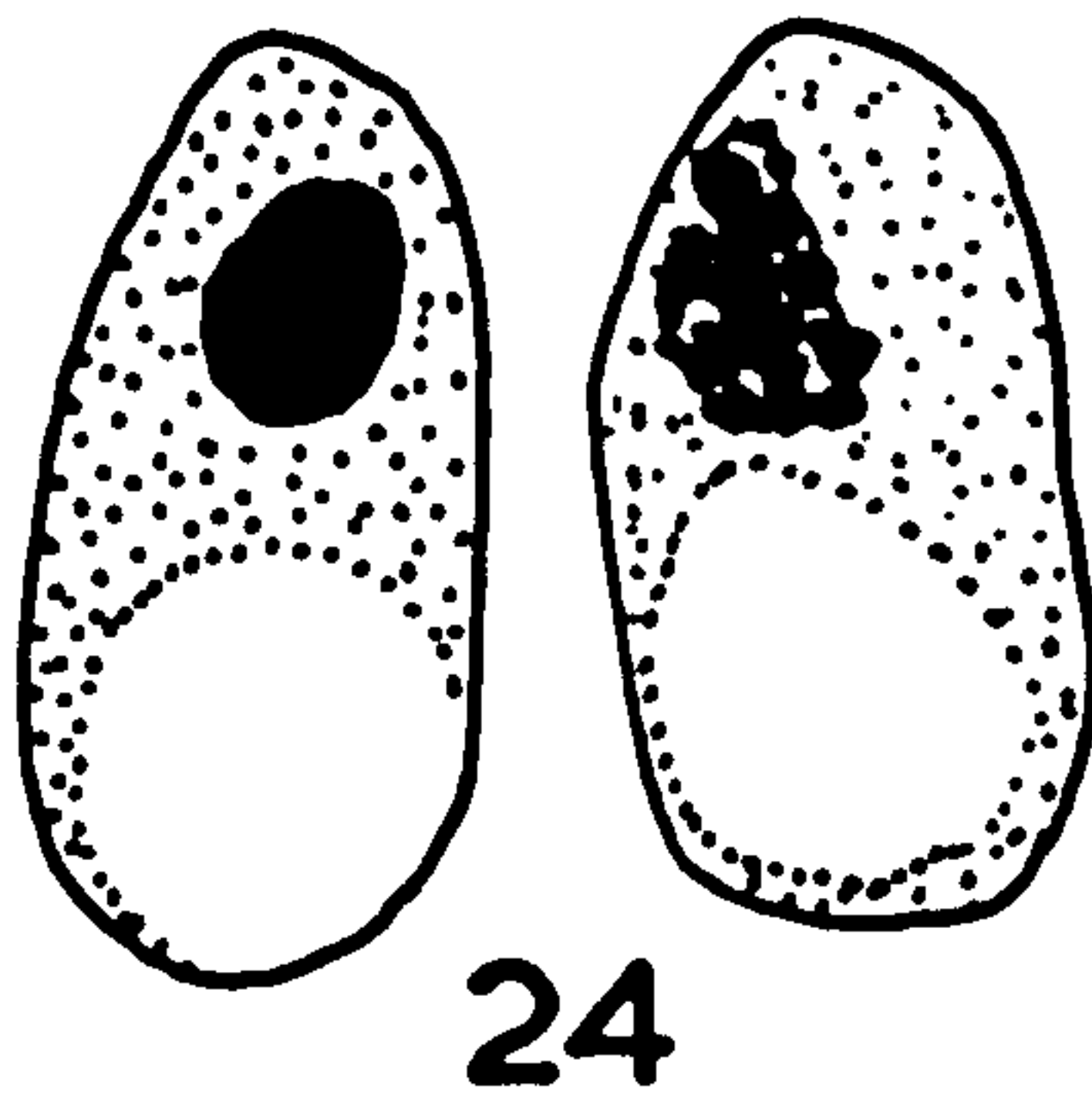
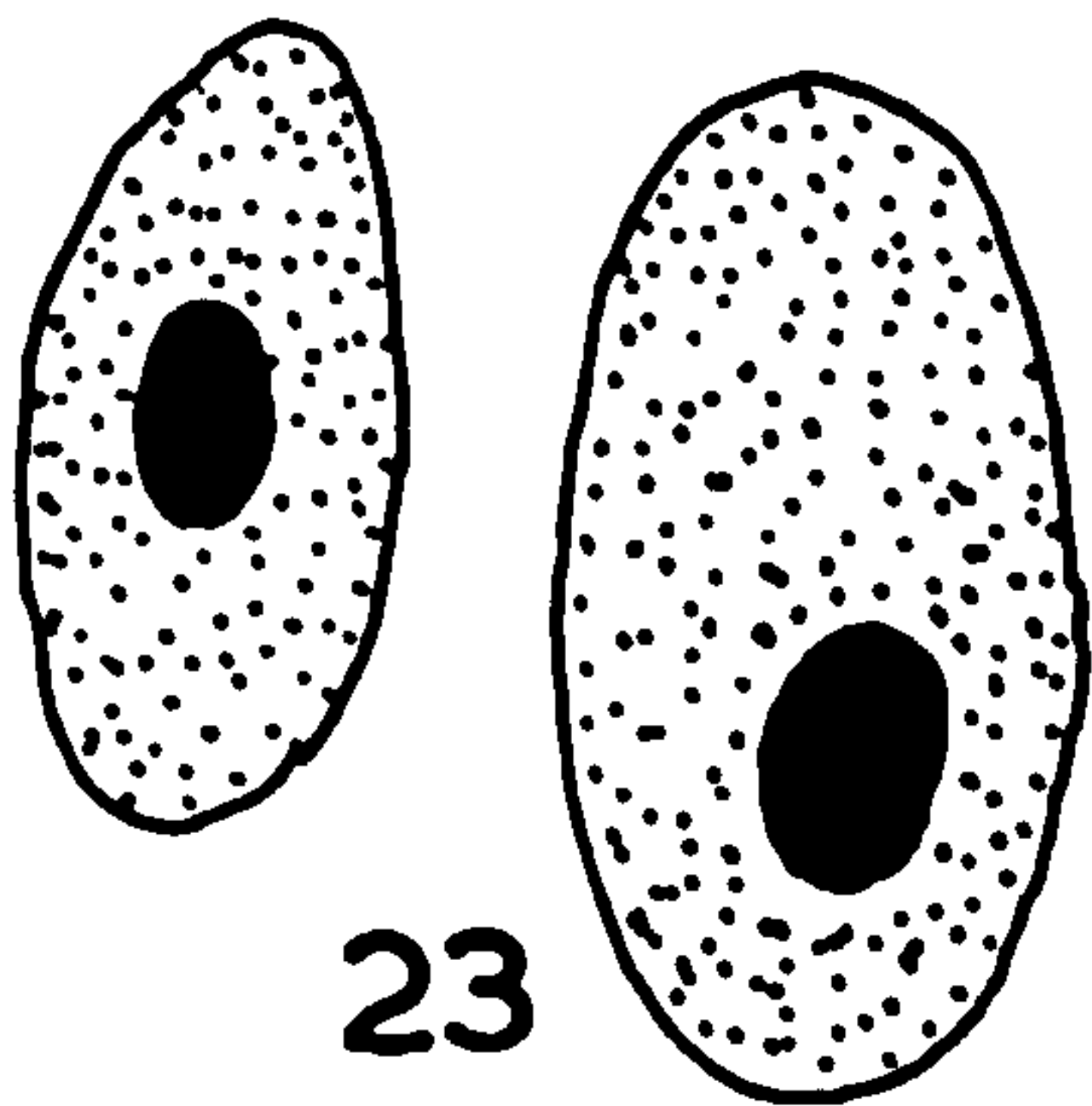
23. Early sporonts.

24. Sporonts showing vacuole at one end.

25-29. Stages in the formation and growth of  
the 'pre-metachromatic granule'.

30. Unusually large sporonts which are probably  
precursors of macrospores.

PLATE V



10  $\mu$ .

Explanation of Plate VI.

Stages in Second-phase Schizogony, from sections stained by the Giemsa-Colophonium method.

31. Early second phase schizonts.

32-33. Nuclear division and elongation .

34. Nuclear separation stages.

35. Stages resulting from cytoplasmic constriction of stages shown in Fig. 34. - Early fusiform schizonts.

36-38. Division of fusiform stages with chain formation, and 'diplocarya' formation.

39. Fusiform schizonts with double nuclei in close association.

40-41. Shortening of processes of fusiform schizonts and deepening of cytoplasm, with fusion of nuclei in some.

42. Sporonts resulting from fusiform schizonts, some with double nuclei others with nuclei fused.

43. Spores with single and double nuclei.



PLATE VI



31



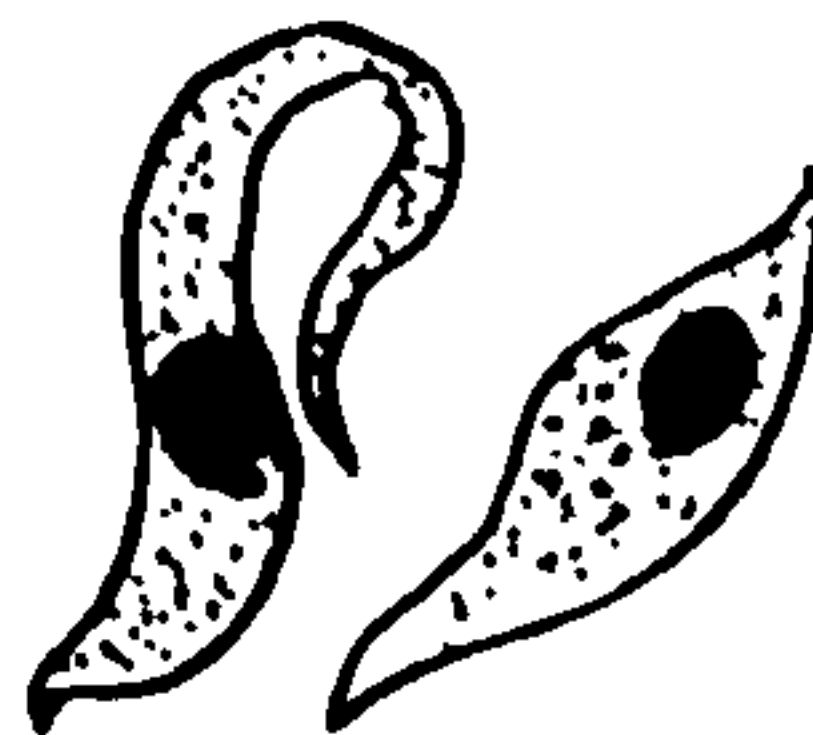
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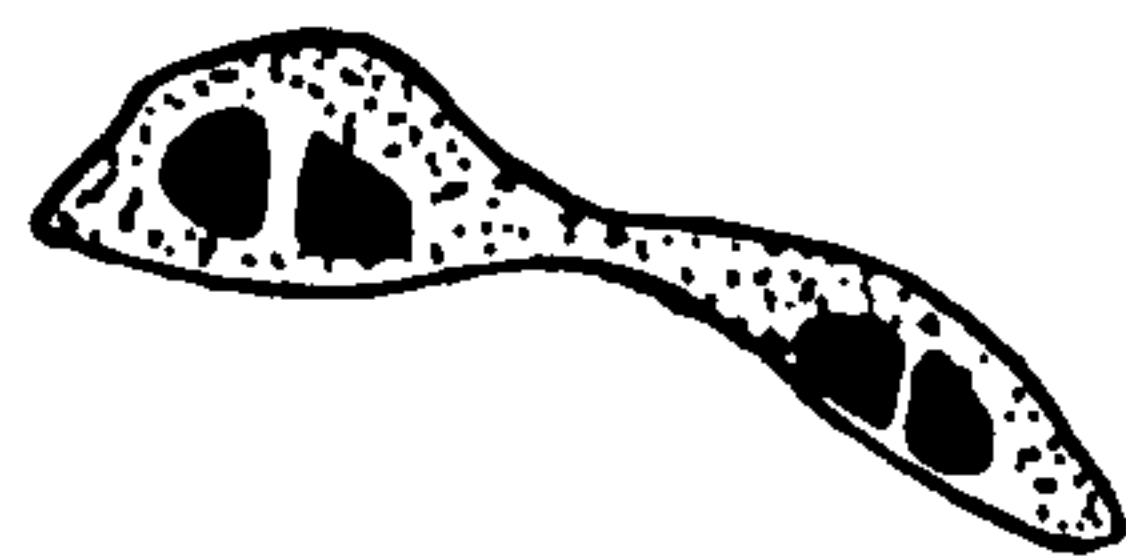
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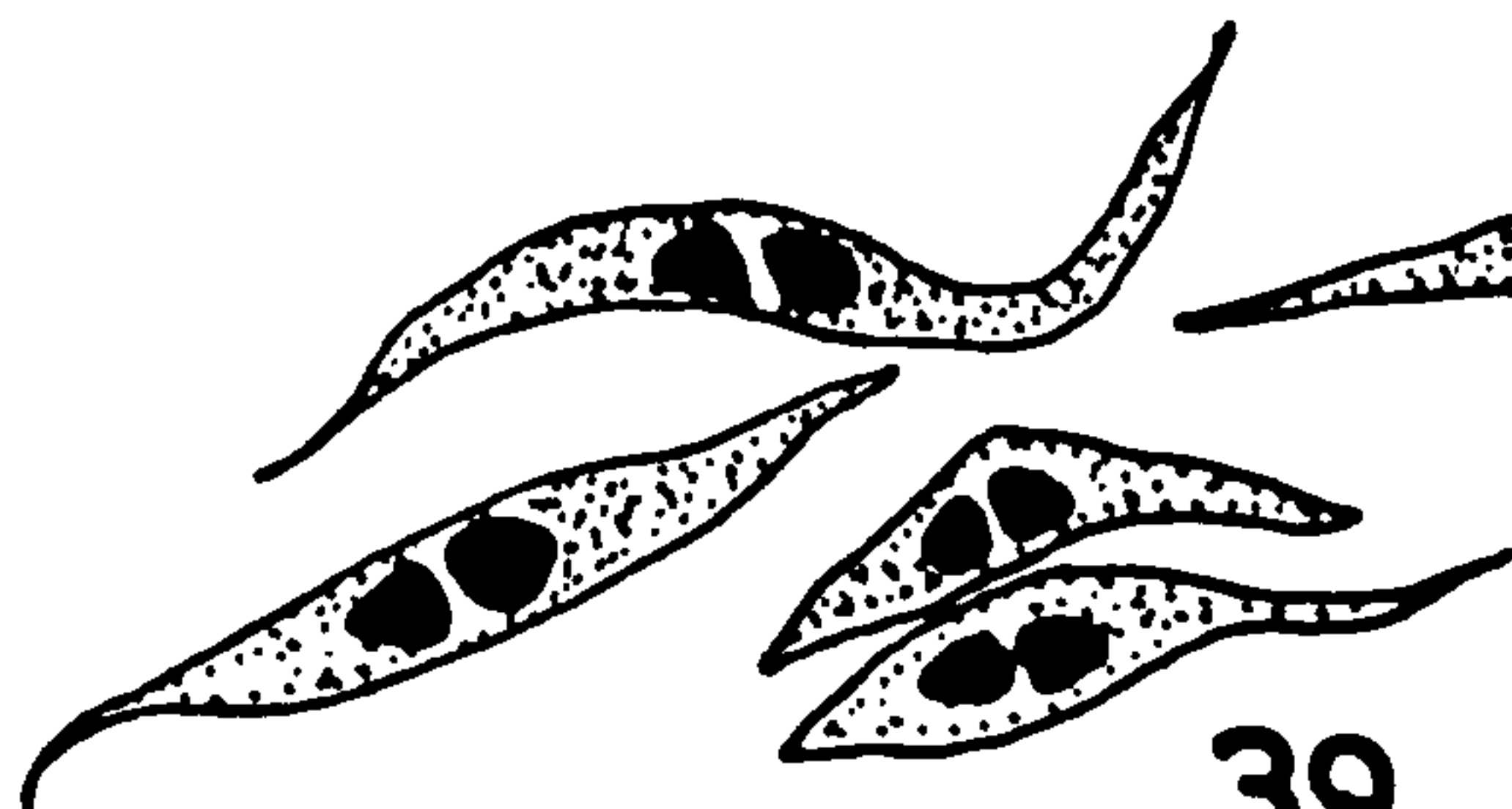
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37



38



39



40



41



42



43

10μ.

These sections were shown to Professor G.R.Cameron F.R.S. of University College, London, who was kind enough to examine them and give me his opinion on these cells. He felt that if they definitely were not stages of the parasite, then their appearances were strongly suggestive of fibroblastic cells met with in vertebrates and higher invertebrates. In a recent demonstration I suggested (Dissanaike 1955 a) that these cells were either fibroblasts or aberrant schizonts of the microsporidian, and that if the former, this would be the first instance of a histopathological reaction in tapeworms. Since then, however, a thorough study of these cells together with an examination of the literature on the microsporidia indicates that these cells are indeed stages of the parasite. A large number of these cells in M 135 were found to have double nuclei which were placed close to one another and were somewhat triangular in outline with the bases opposed, and this was more strikingly seen in M 125. The nuclei stained faintly Feulgen positive.

Another interesting fact that soon revealed itself was that the worms that show these fusiform cells are the very ones that show macroscopic white opaque



areas of infection and also that the spores from these areas are smaller and sometimes rounder than usual. And in sections of these two worms it was seen that the smaller spores were associated with the fusiform cells. It was now evident that where rapid multiplication of the parasite occurred, the pre-sporont stages which were not easily made out normally, took on this fusiform appearance for some obscure reason, and that the sporonts that resulted from them were smaller than normal and hence the spores were of a smaller size. As will be seen later, in support of this is the fact that these smaller spores appear in areas that are heavily packed with spores and that this overcrowding is responsible for the small size of the spores.

The fusiform cells were seen best in W 125 where all stages from the primary phase schizonts to spindle-shaped uninuclear cells followed by bi-nucleate fusiform cells were recognised. (Plate VI, FIG. 32-33). It is clear from this that once spore formation is about to begin the schizonts commence to elongate (Plate VI, Fig. 32,33), their nuclei divide and at first they get separated widely and the cytoplasm constricts between them (Plate VI, Fig. 34) and this process



can go on till a chain of these schizonts is formed . Soon the nucleus of each of these cells divides into two but they do not separate ( Plate VI, Fig. 36-39 ). They remain closely associated forming "diplocarya" of Debaisieux . The fusiform cells at this stage vary in length from 7.5  $\mu$ . to 17  $\mu$ . in sections , and at first are 1.4-1.5  $\mu$ . wide but soon increase in width to about 2  $\mu$ . The cytoplasm of these fusiform schizonts which at first is very faintly staining becomes more and more deeply staining and vacuolated ( Plate VI, Fig. 40, 41 ). The cells as they widen lose their processes and become at first diamond-shaped and later ovoidal measuring 6-7.5  $\mu$ . in length with denser more vacuolated, purplish staining cytoplasm. The nuclei during the whole of this process remain closely associated and gradually come in contact with each other, and in some , by the time the ovoidal stage is reached, they fuse into a single nucleus which has a characteristic appearance( Plate VI, FIG.41,42). The ovoidal stages are clearly sporonts which soon transform into spores.(plate VI , Fig. 42, 43 ).

This second phase of schizogony then has given rise to elongate sporont mother cells whose nuclei though divided do not separate , but fuse sooner

or later during sporogony. There can be little doubt from these observations that autogamy takes place in this species. The significance of this process will be discussed later. It appears that these fusiform cells with elongate processes are not the usual precursors of the sporonts as is seen for instance in M 35, and in fact in all the worms where the spores are less crowded and as a result larger in size. Here the sporont precursors are seen as faintly staining ovoidal cells (Plate V, Fig. 23) resulting from the later primary phase schizonts. These stages are so rare in sections that a detailed study of the nuclear changes in them is not possible, but it is likely that a similar nuclear division followed by an immediate fusion of these nuclei occurs, since a few sporonts of this type are seen to possess double double nuclei.

It is not easy to explain why this second phase of schizogony takes on different patterns in different worms, and why the fusiform cells that appeared in M 125 and M 135 possess such long processes. It is of course possible that the continued contraction and relaxation of the worms can account for this morphological character. In many of the areas



where these fusiform cells occur they seem to be present in the region of the well developed longitudinal muscle bundles of the parenchyma, and, as will be seen later, in one of the experimental infections of Hymenolepis nana of mice with this parasite these fusiform cells appear in the scolex and neck regions where the activity of the worm may be considered to be quite marked.

#### Sporonts

In those worms that <sup>show</sup> the larger spores which are less crowded together, the schizonts soon give rise not to daughter schizonts but to the precursors of spores - the sporonts. These uninuclear results of schizogony become ovoidal with a centrally placed nucleus which is more or less compact in sections but appears to be made up of granules of chromatin in smears where it shows an irregular outline due to flattening (Plate V, Fig. 24-30). The cytoplasm soon darkens and a vacuole appears at one end due to the cytoplasm receding from that end (Plate V, Fig. 24). These sporonts measure 4.8-6.5  $\mu$ . by 2.1- 3.0  $\mu$  in dry-fixed smears. Occasionally much larger sporonts measuring 8- 9.5  $\mu$  by 4  $\mu$ . are seen (Plate V, Fig. 30) which are probably the precursors of macrospores. What stimulates the



production of these sporonts and puts an end to schizogony in these cases is not clear. For instance in places where there are a large number of parasites in one spot there may be all stages from schizonts to sporonts and spores in the same situation, whereas in a few worms the infection is so scanty (M 35) that no more than about half dozen stages are present in one spot in a section. In the latter instance it is not uncommon to find several sporonts and one or two spores but no schizonts. This means that spore formation is not induced by overcrowding with stages of the parasite as is generally believed to be the case in microsporidia. It may on the other hand be due to some reaction on the part of the host or a change in its internal environment.

In worms in which the fusiform schizonts appear the sporonts are formed from these elongate cells by shortening of the processes and the cytoplasm becoming more dense. As was mentioned before, these sporonts may have either one or two nuclei according to whether the autogamous process was completed or not.

The subsequent development of the sporonts of the two types is the same and the description that

follows applies to both. In polysporous microsporidia where a single sporont cell eventually gives rise to more than one spore, the sporont first divides into as many sporoblasts. In the genus Nosema, to which the present species belongs, each sporont gives rise to a single spore and so the terms sporont and sporoblast are synonymous. It is therefore preferred to omit the latter term from the description.

The sporont as mentioned before has generally a single nucleus placed at the middle of the ovoidal body. As seen earlier a clear space soon appears at one end and the nucleus recedes to the other. The appearances of the sporonts at this stage are best seen in smear preparations ( Plate V, Fig. 23-30). In fresh smears examined under phase contrast or in Toluidine blue (Plate VII, Fig. 49 and 52), the sporonts are seen to possess a narrower anterior end, and at the broader end a very distinct granule soon appears within a vacuole (Fig. 5). This granule stains a reddish colour in Toluidine blue and increases in size as the sporont transforms into a spore. Both in Toluidine blue preparations and in smears examined under phase contrast there appears a darker area towards the anterior end of the sporont.



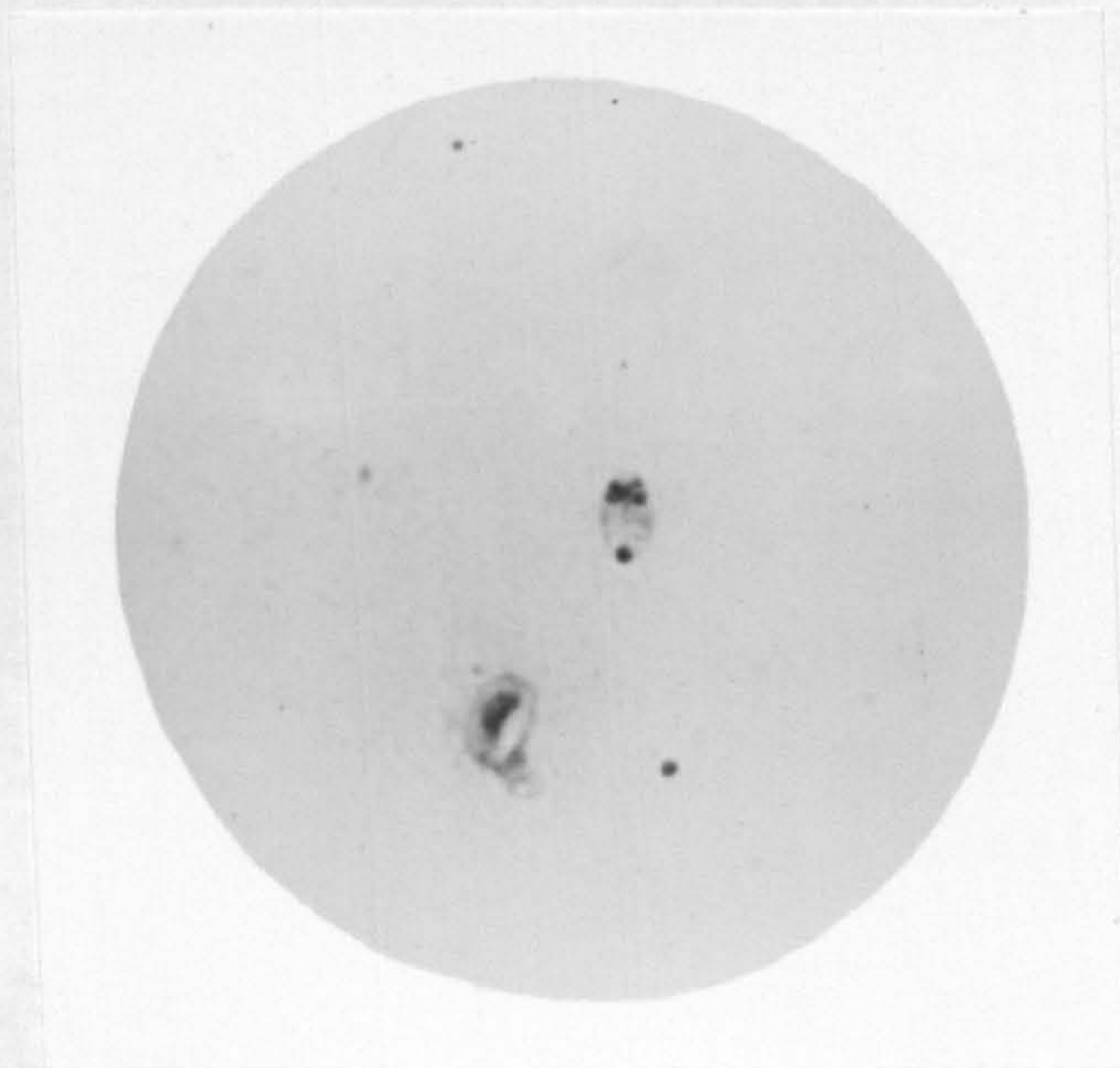


Fig E.

Sporont from a smear of Moniezia expansa  
showing "pre-metachromatic granule".

( Giemsa stain) X1200.



which stains a bluish colour in Toluidine blue and corresponds to the position of the nucleus (Plate VII, Fig. 49,52). In Giemsa stained smears these sporonts (Fig. 49,52) show many more details of structure. Here the earliest stages show only a clear vacuole at the broad end. Then a deeply staining purplish granule appears in this vacuole which no doubt corresponds to the granule seen in fresh preparations examined under phase contrast and after staining in dilute Toluidine blue. As the further development of the sporont into the spore shows this granule increases in size and becomes the metachromatic granule in the mature spore. I therefore propose to refer to this granule as the "pre-metachromatic granule". This structure has not hitherto been described in the microsporidia, but a few observers seem to have pictured it in the sporonts although they have not referred to it in any detail. For instance in Thelohanias chaetogastri Schröder (1903) describes and pictures the nuclei at the posterior end of the sporoblasts surrounded by a vacuole, while in the middle of the sporoblast he shows a deeply staining oval body. It is possible that he mistook the posterior body to be the nucleus whereas the true nucleus was the deeply staining body at the centre. Again Kudo (1925) in Stempellia

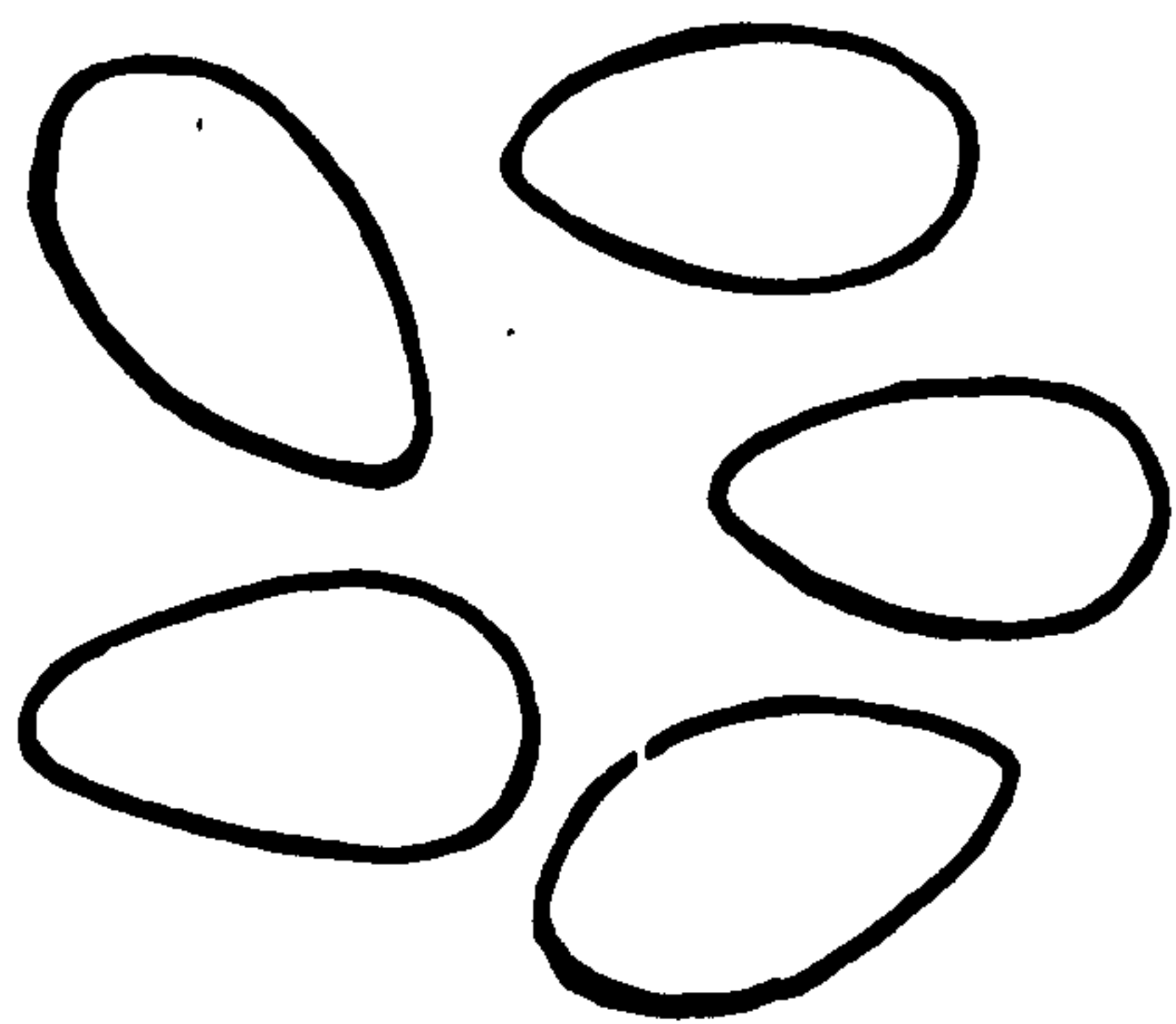
## Explanation of Plate VII

### Spores from unfixed preparations

- 44 & 45. Outline drawings of spores from two infected worms one (45) of which showed opaque areas macroscopically.
- 46. Spores from a saline preparation showing a group of 6 spores within a membrane.
- 47. Two double spores.
- 48. A double spore with filament extruded from one end.
- 49. Sporonts under phase contrast.
- 50-51. Spores with extruded filaments under phase contrast.
- 52. Sporont in saline stained in dilute Toluidine blue.
- 53+ Fresh spores stained in dilute Toluidine blue.
- 54. Two spores after treatment with 66% nitric acid showing the so-called 'polar capsule'

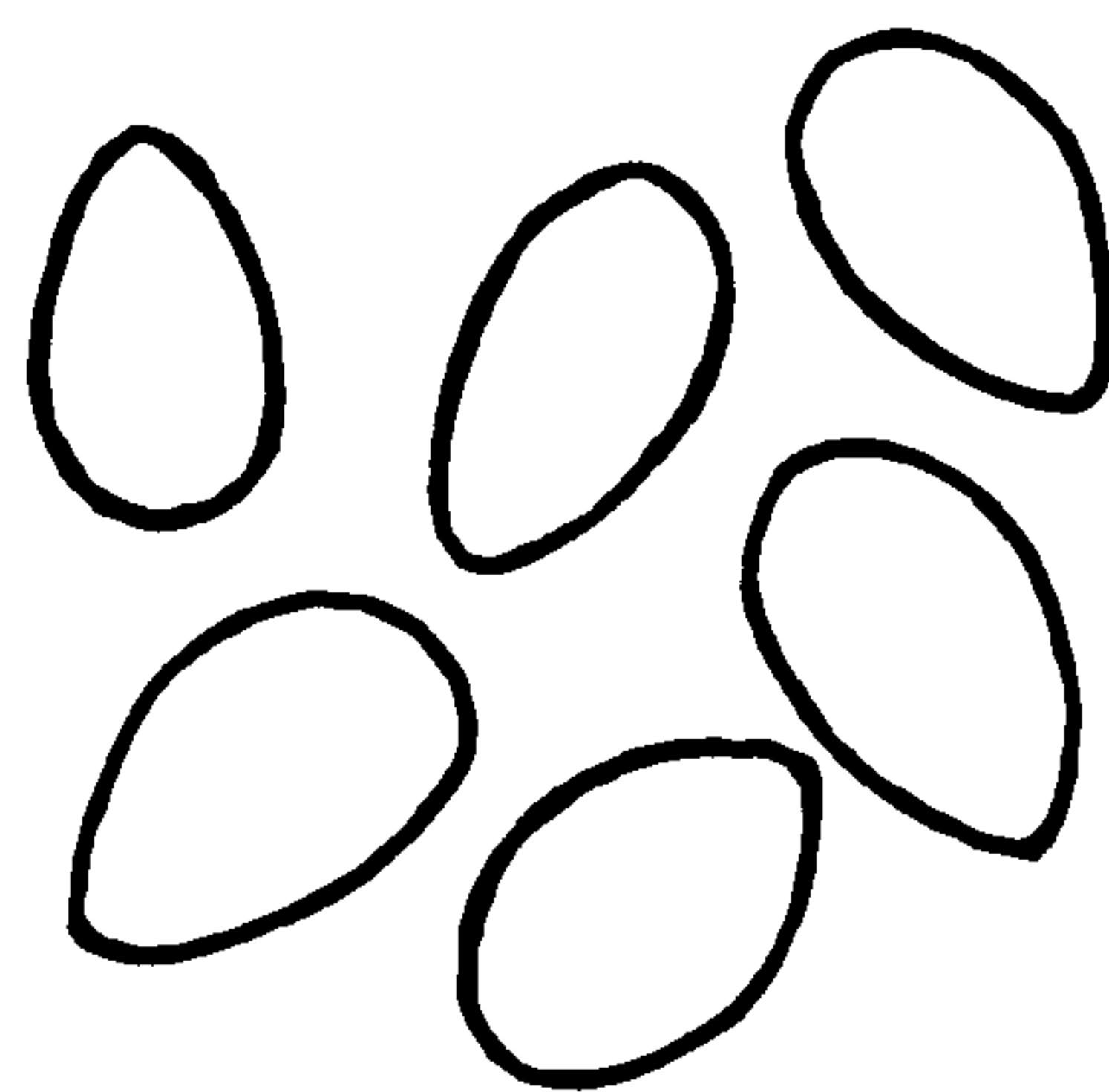


PLATE VII

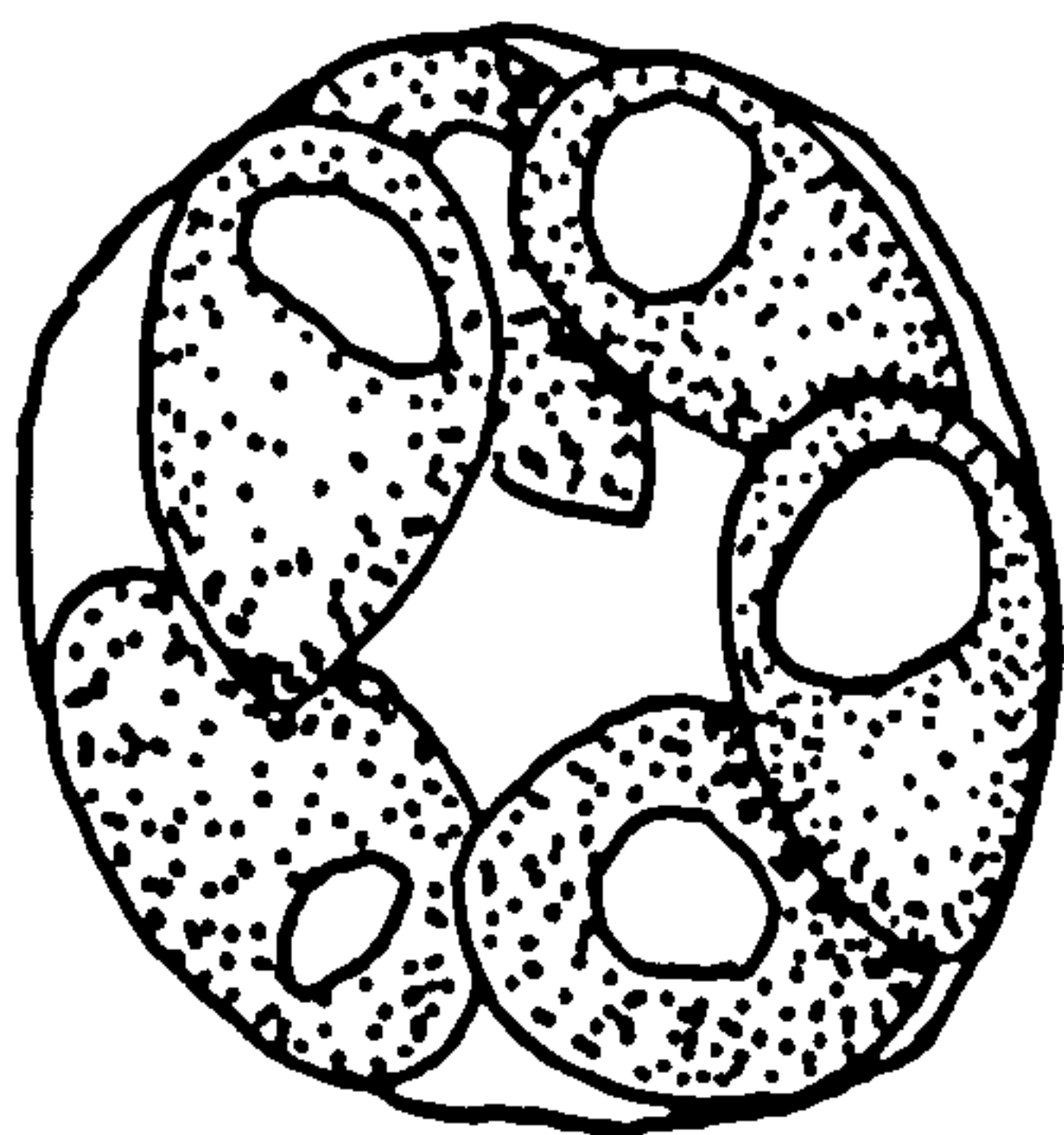


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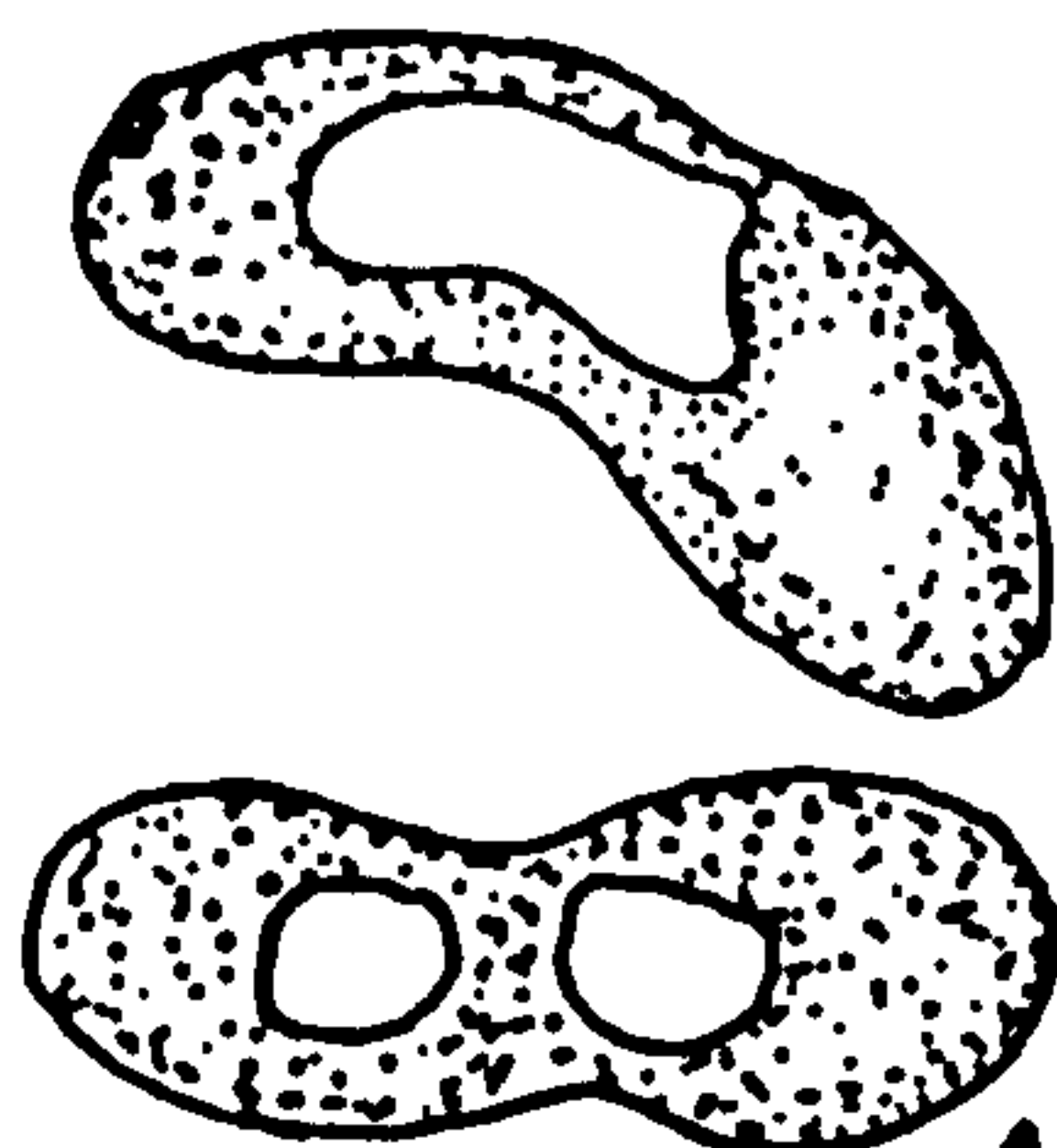
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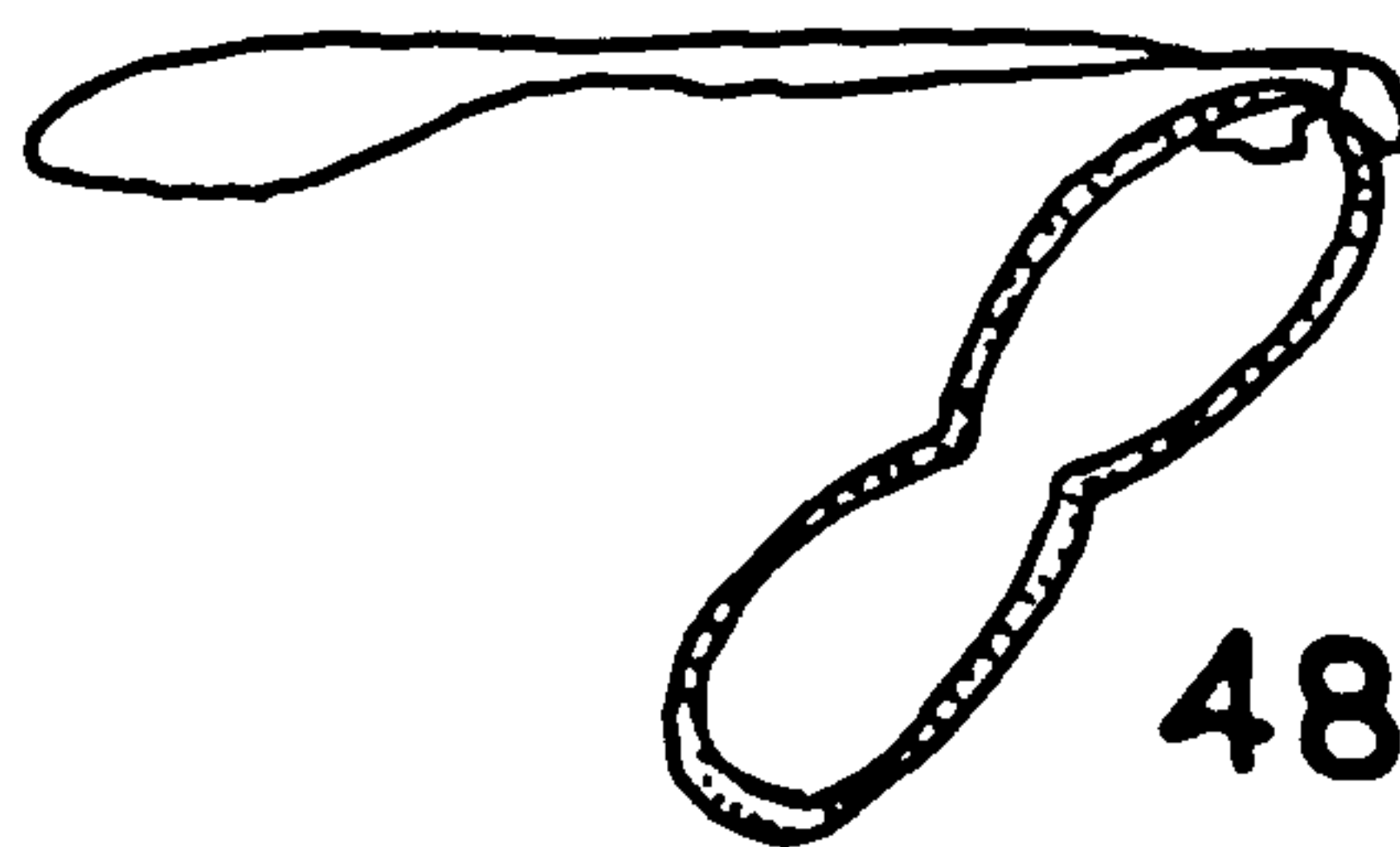


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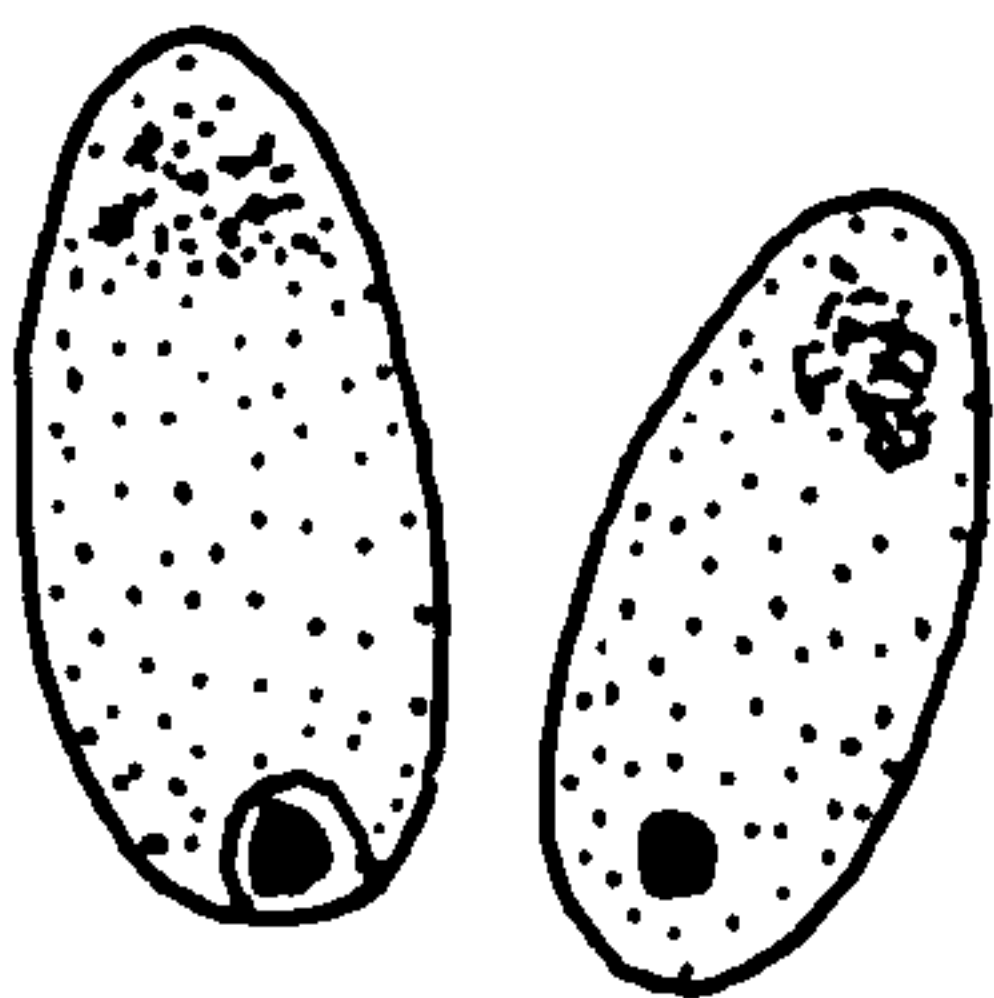
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48

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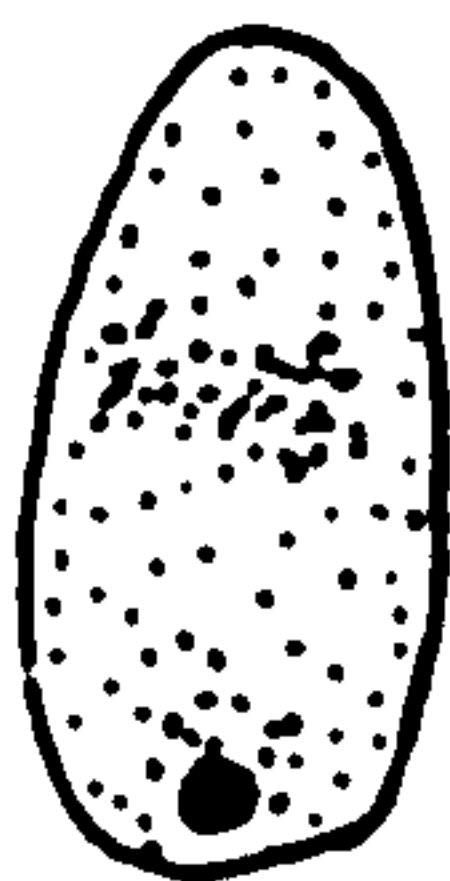
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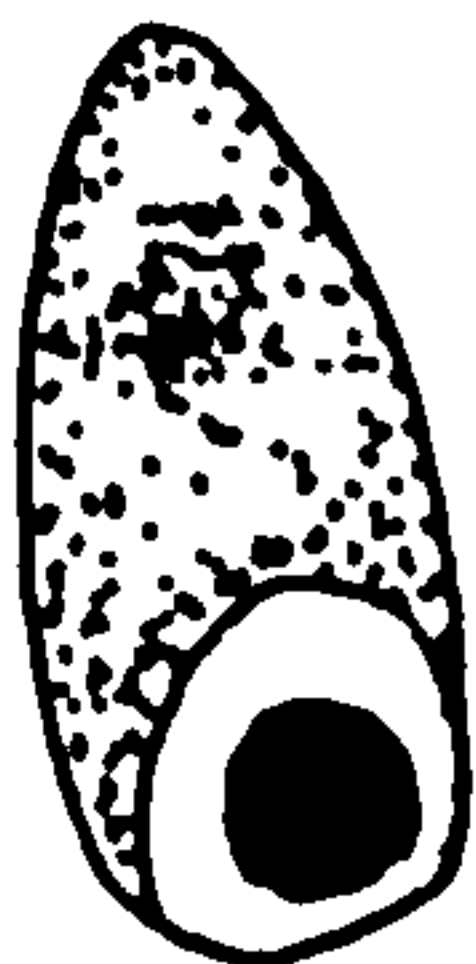
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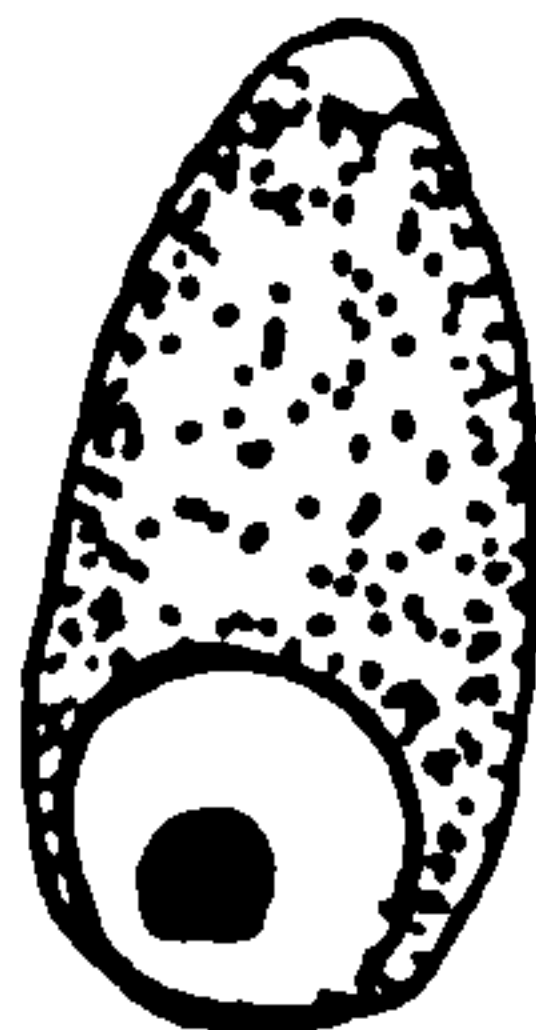
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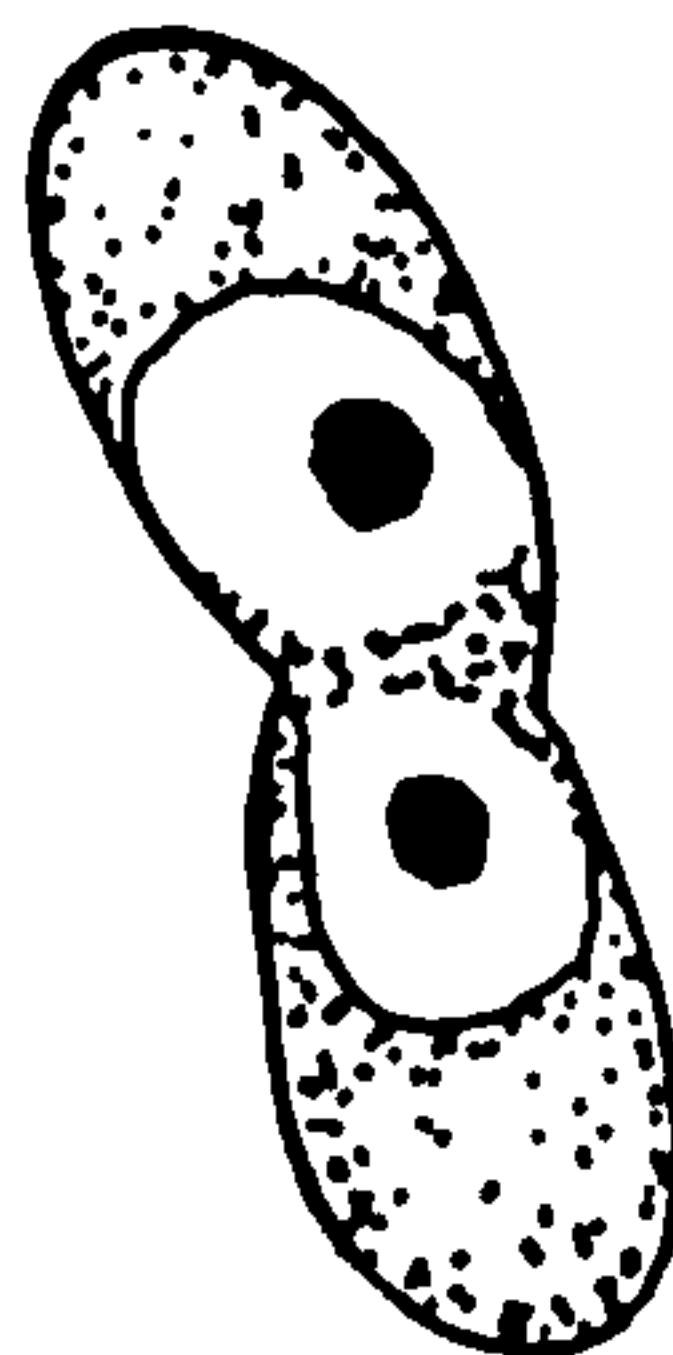
52



53



54



55

10μ.

nanna describes deeply staining granules in a clear space at one end. of the sporoblasts. According to him these granules become smaller in size and larger in number as the filament is formed which he suggests is an indication that they are probably used in the formation of the filament. Whatever the true significance of these granules it is certain that in Nosema helminthorum at any rate they are definite and characteristic structures which are represented in the mature spore by the metachromatic body.

Although in sections of N 123 and 135 some sporonts had two nuclei due<sup>to</sup> the delay in fusion, smears from these worms showed sporonts with only a single nucleus, although one sporont was seen with an appearance that could be interpreted as fusing nuclei ( Plate V, Fig. 25).

### Spores

The fresh spores of N. helminthorum ( Plate I, Fig. 1) are characteristically ovoidal or egg-shaped with a narrow pointed anterior pole and a broader posterior pole, where a clear space is invariably present which is generally referred to as a vacuole. The vacuole, which occupies about a third of the



length of the spore, is in many instances placed asymmetrically to one side as is seen in Fi. 1. In a few spores another clear space may be seen at the anterior end. The surface of the spore is smooth and there is no evidence of a bivalve structure as has been described for some of the microsporidian spores like Glugea anomala and Thelohania giardi (Thélohan 1895) Plistophora simulii (Lutz and Splendore 1904), and Thelohania opacita (Kudo 1924). As to the surface of the microsporidian spore it is generally considered to be smooth and structureless, but in two instances certain markings have been observed. Thélohan (1895) noticed longitudinal striations on the surface of the spores of Thelohania giardi and Doflein (1893) found similar markings in Gurleva tetraspora. The recent demonstration by Krieg (1955) that the spore of Plistophora melolanthae shows under the Electron microscope "eine deutliche Riffelung" suggests that similar ridges may be made out in all microsporidian spores when viewed under such high powers.

The fresh spores generally measure 5.81 - 6.8  $\mu$ . by 3.25- 3.85  $\mu$  with an average value of 6.25 by 3.5  $\mu$ . But in very heavy infections there appear all types of abnormalities. The commonest abnormality is the double



spore (Plate VII, Fig. 47,48,54 ) which is apparently formed by the rapid and precocious formation of the spore wall before schizonts (binuclear ) have had time to transform into sporonts. In these double spores the union is obviously by the broader ends, as is seen in Toluidine-blue-stained preparations where the metachromatic body, which is situated in the broader end of single spores, is placed adjacent to its partner (Plate VII, Fig. 54). Furthermore, as shown in Fig. 48 these spores possess a filament and probably also a sporoplasma, the filament being extruded from the end corresponding to the usual narrow end. Another peculiarity of the spores in some worms, which may be considered an abnormality due to rapid multiplication of the parasite and to overcrowding, is the appearance of spores of a smaller size which are at the same time either rounder or narrower than the normal spores. Some of these spores are drawn side by side with the normal spores for comparison (Plate VII, Fig. 44 and 45) and though there is a definite difference in their dimensions ( Average values being respectively  $6.5 \mu$  by  $3.5 \mu$  , and  $5.3 \mu$  by  $3.2 \mu$  ) they cannot be regarded as belonging to different species for several reasons. First, wherever these smaller spores appear they are associated with macroscopically visible

opaque white areas in the infected worms , and in sections these areas show marked concentration of spores. In addition the smaller type of spore occurring in different worms does not always show the same dimensions, for instance in one such worm the average dimensions of fresh spores were  $5.25 \mu$ . by  $3.2 \mu$  while in another the values were  $5.6 \mu$  by  $2.9 \mu$  . This shows that the size of the smaller spore is not consistent, but varies according<sup>n</sup> the conditions under which it has developed.

Some interesting studies were made on fresh spores under phase contrast and after vital staining with dilute Toluidine blue. The mature spores show no more details than can be <sup>made</sup> out by ordinary methods, but the immature spores show the following details. As in the sporonts they <sup>show</sup> a granule placed in the posterior vacuole which is of a larger size. (Plate VII , Fig. 53 ). The region of the nucleus also presents itself as a darker area near the anterior end , which stains blue in the Toluidine blue preparations.

A few spores with filaments extruded show a bluish stained mass at the anterior end which is probably the sporoplasm that was left behind when the filament was extruded.



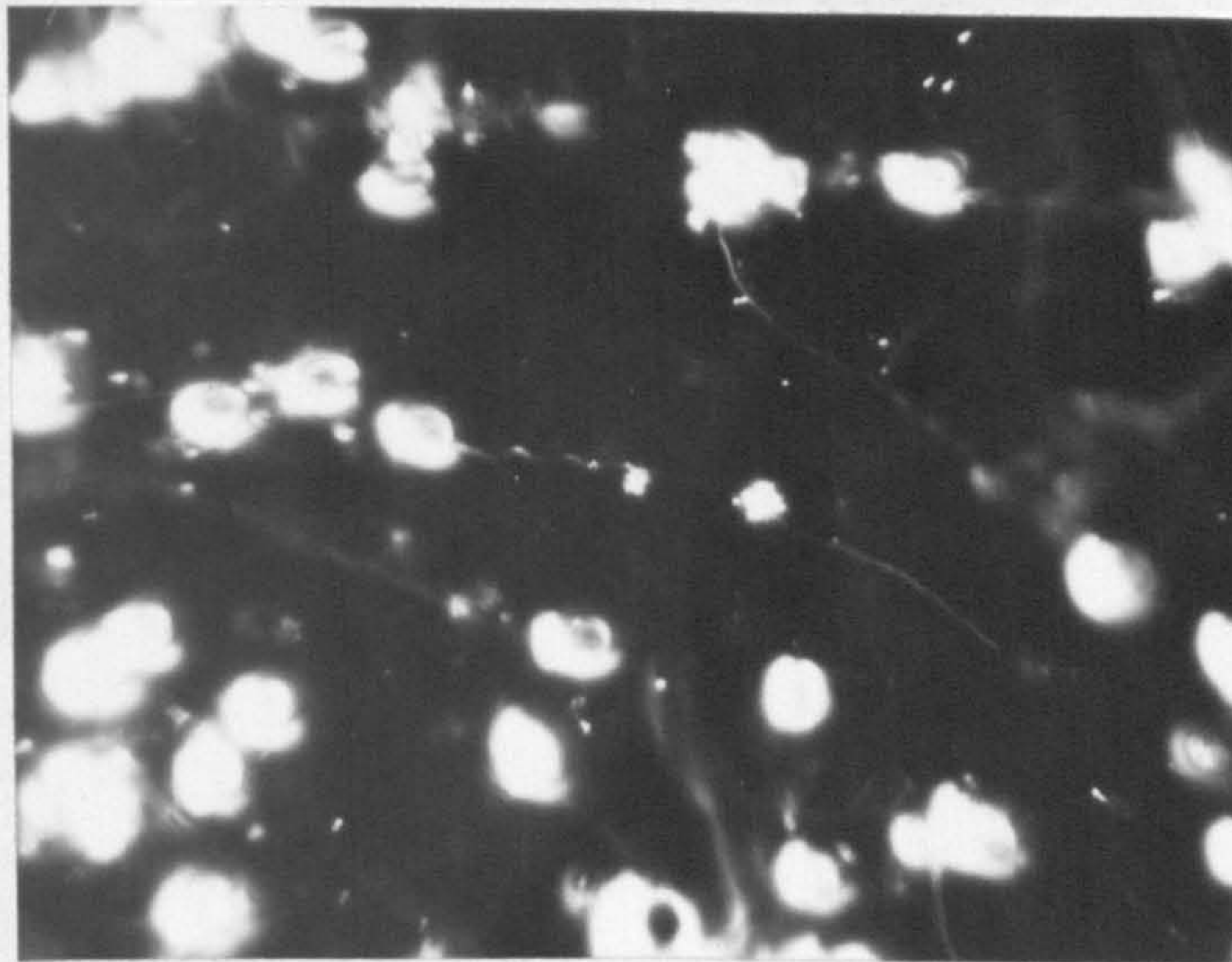


Fig. F.

Spores under dark-ground illumination showing  
extruded filaments ( X 750 )

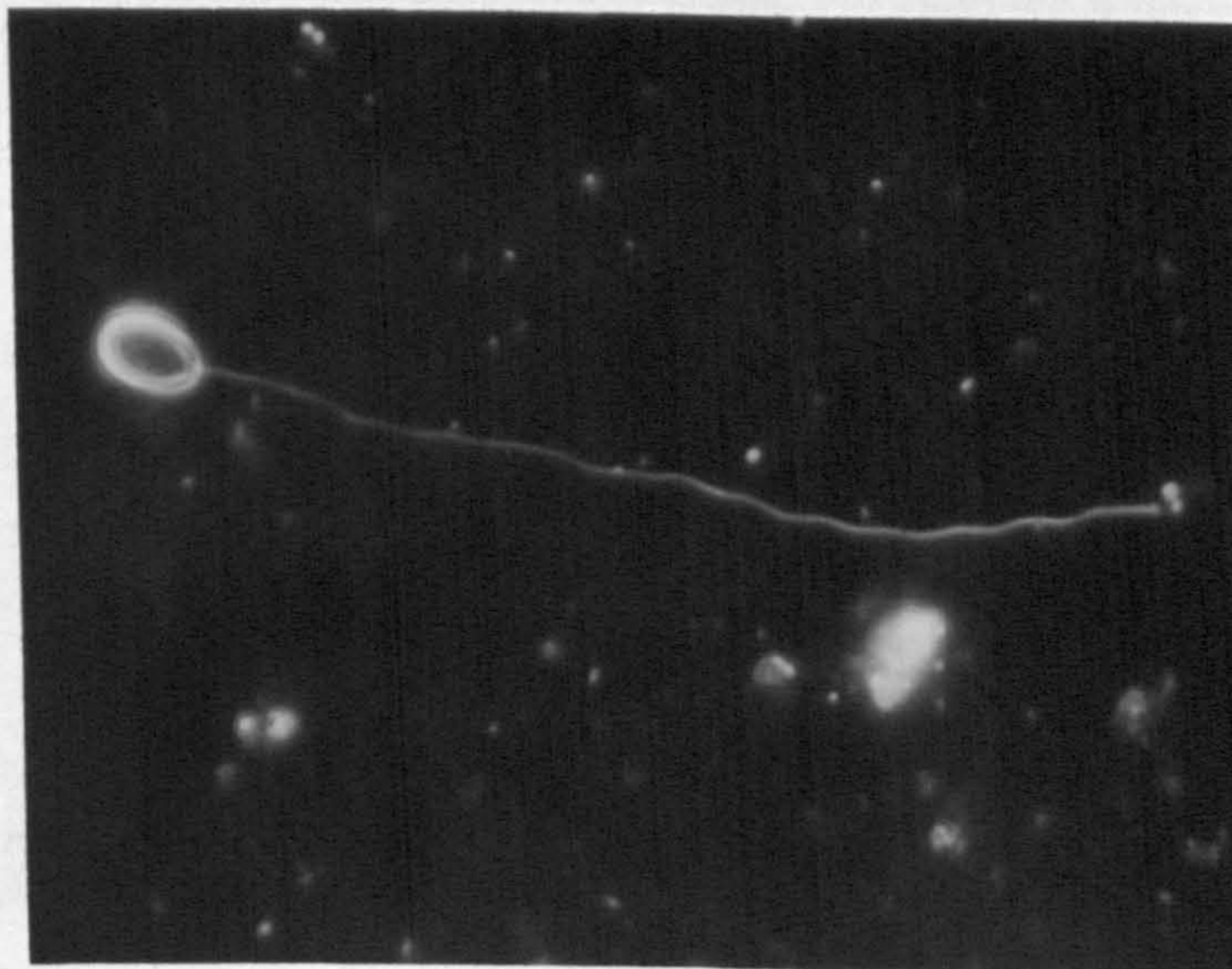


Fig. G.

A single spore with extruded filament under  
dark-ground illumination. ( X 1250. )



Although a large number of methods for producing extrusion of filaments in microsporidian spores were employed, none gave any results except the mechanical pressure method of Kudo. This was invariably successful and was used for studying spores under dark-ground illumination. In another method the spores were kept in saline under a sealed cover-slip overnight; on the following day a large number showed extruded filaments either still attached to the empty spores or detached from them. The following reagents were used without success: - glycerine, dilute nitric acid, dilute acetic acid, Gram's iodine, Hydrogen peroxide, Iron alum(4 %) , tap water and distilled water.

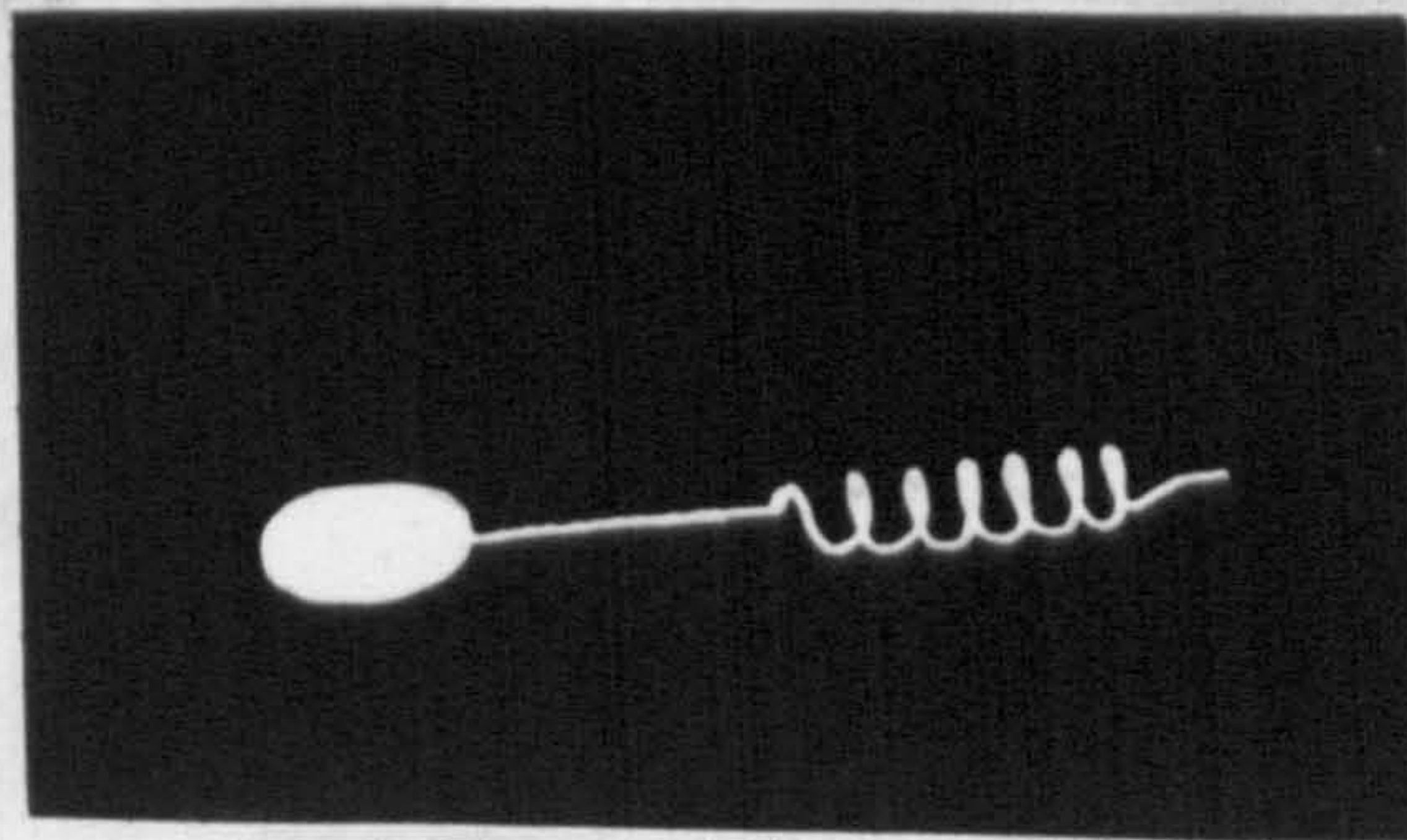
A method recently suggested by Gibbs (1953) namely adding water to dried spores, was found to give good results although his observations regarding the evagination of the filament were not confirmed.

Finally a method which gave not only a high percentage of extruded filaments but also some useful information on the mode of emergence of the sporoplasm will be discussed later.

Spores examined by dark-ground illumination after extrusion of filaments showed some interesting



features ( Fig. F and G ) . In a large number of them the filament , as soon as it is extruded, has 6 to 8 coils with a straight portion near the attached end ( Text Fig. 1) This appearance of the filament that has just been extruded gives a clue to the internal arrangement of the filament in the spore as will be discussed later. After a few minutes these coils



Text Fig. 1.

straighten out and the filament is seen as a straight thread. In none of the dark-ground preparations was a body observed at the tip of the extruded filament that might be taken for the sporoplasm. This is probably because the method for extrusion is so violent that the sporoplasm is torn away from the filament.

On more than one occasion fresh smears revealed two or more spores within cyst-like structures . One of these containing 6 spores is shown in Fig. 46 (Plate VII)



This appearance is clearly due to several spores being enclosed in a common envelope formed by the destruction of the host tissue and gives a false impression that the spores are formed from the same pansporoblast. It was probably this fact that led Labbé (1899) to place this parasite in the genus Plistophora. The presence of groups of spores in cystic spaces in the host tissue is also noticed in sections, but, as mentioned before the development of the spores always takes place by a sporont giving rise to a single spore and therefore there can be no doubt that this species belongs to the genus Nosema.

Appearance of spores in stained preparations - Wet and dry-fixed preparations of spores were quite unsuitable for study of the internal structure, especially those stained in Heidenhain's iron haematoxylin which produces a number of artefacts. Some of the dry-fixed smears stained in Giemsa stain, however, though showing considerable shrinkage of the contents of the spores, are helpful (Plate VIII, Fig. 64). They show a deeply staining purplish red spot at one pole which corresponds to the position of the metachromatic body, and a reddish area more anteriorly placed representing the nucleus. On the whole, sections of infected worms are more

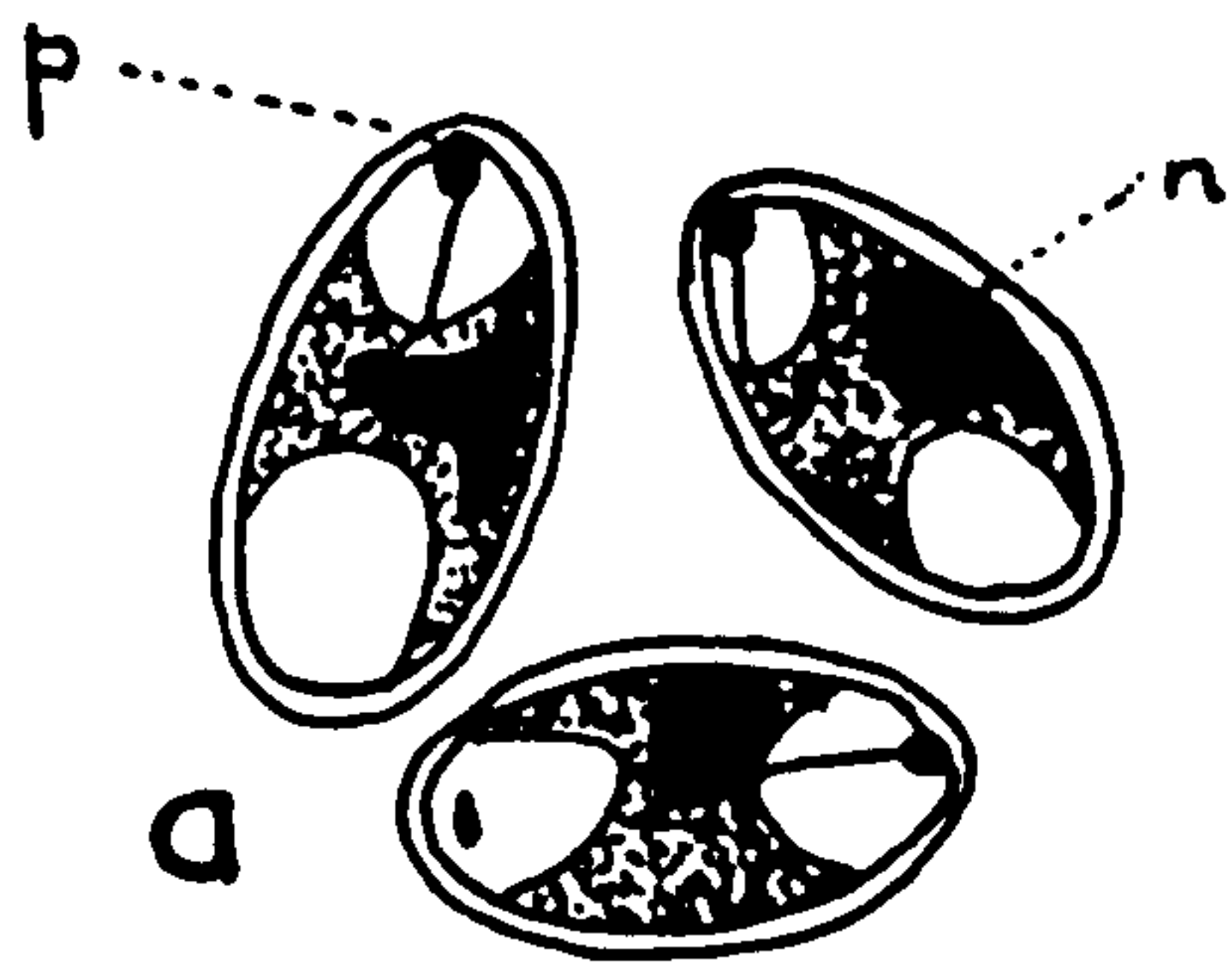
## Explanation of Plate VIII

### Spores from stained preparations

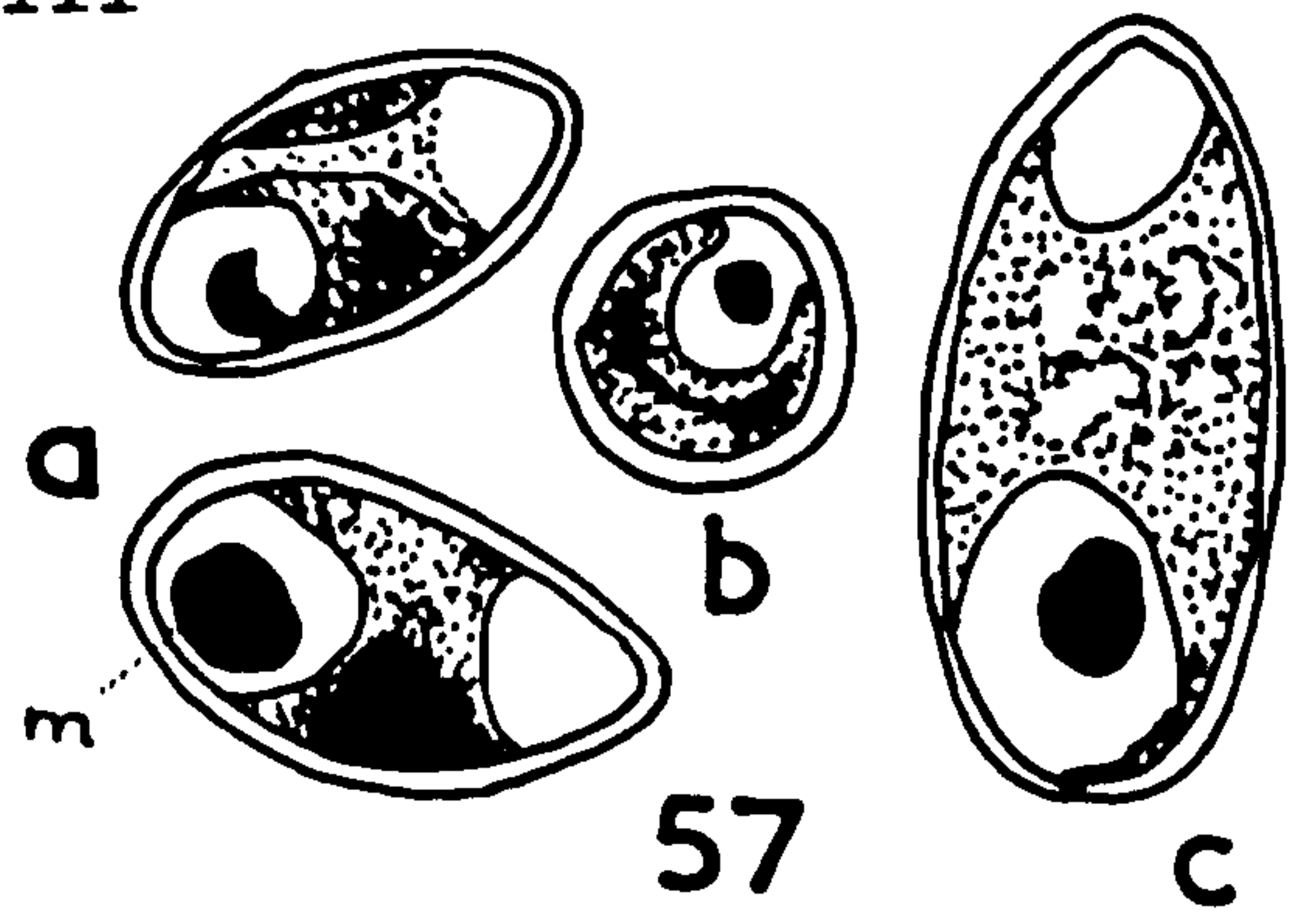
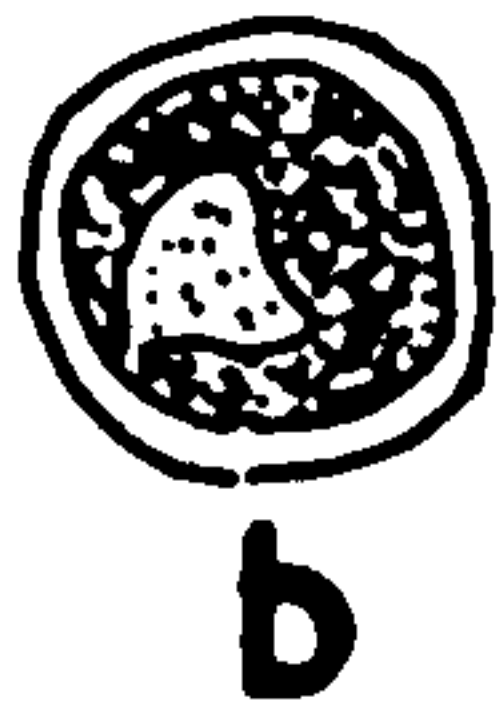
56. Spores stained in Heidenhain showing girdle-shaped sporoplasm, nucleus(n), and polar filament attachment (p).
57. Spores stained by Maximow's method. a- showing metachromatic body (m). b- spore in T.S. showing c- a macrospore. (annular sporoplasm.
58. Spores stained in Giemsa in L.S. and T.S.
59. " " " but overdifferentiated showing nuclei clearly and filaments ? (f).
60. Two spores from M. 125 showing single and Double nuclei.
61. Spores from sections stained in Feulgen Reagent.
- 62.& 63. Spores from sections of M135 and M 126 respectively.
64. Two spores from a dry-fixed smear stained in Giemsa stain.



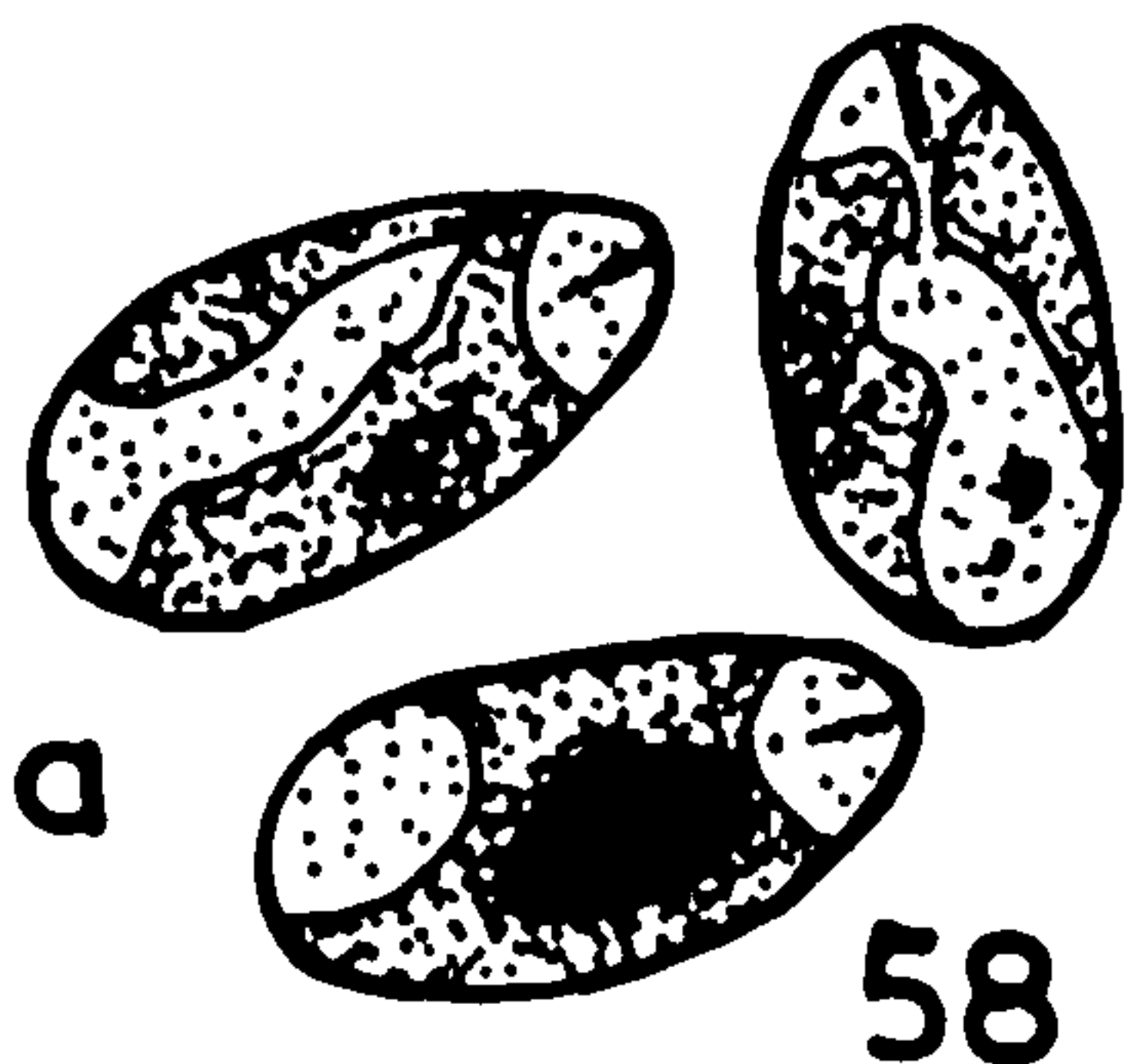
PLATE VIII



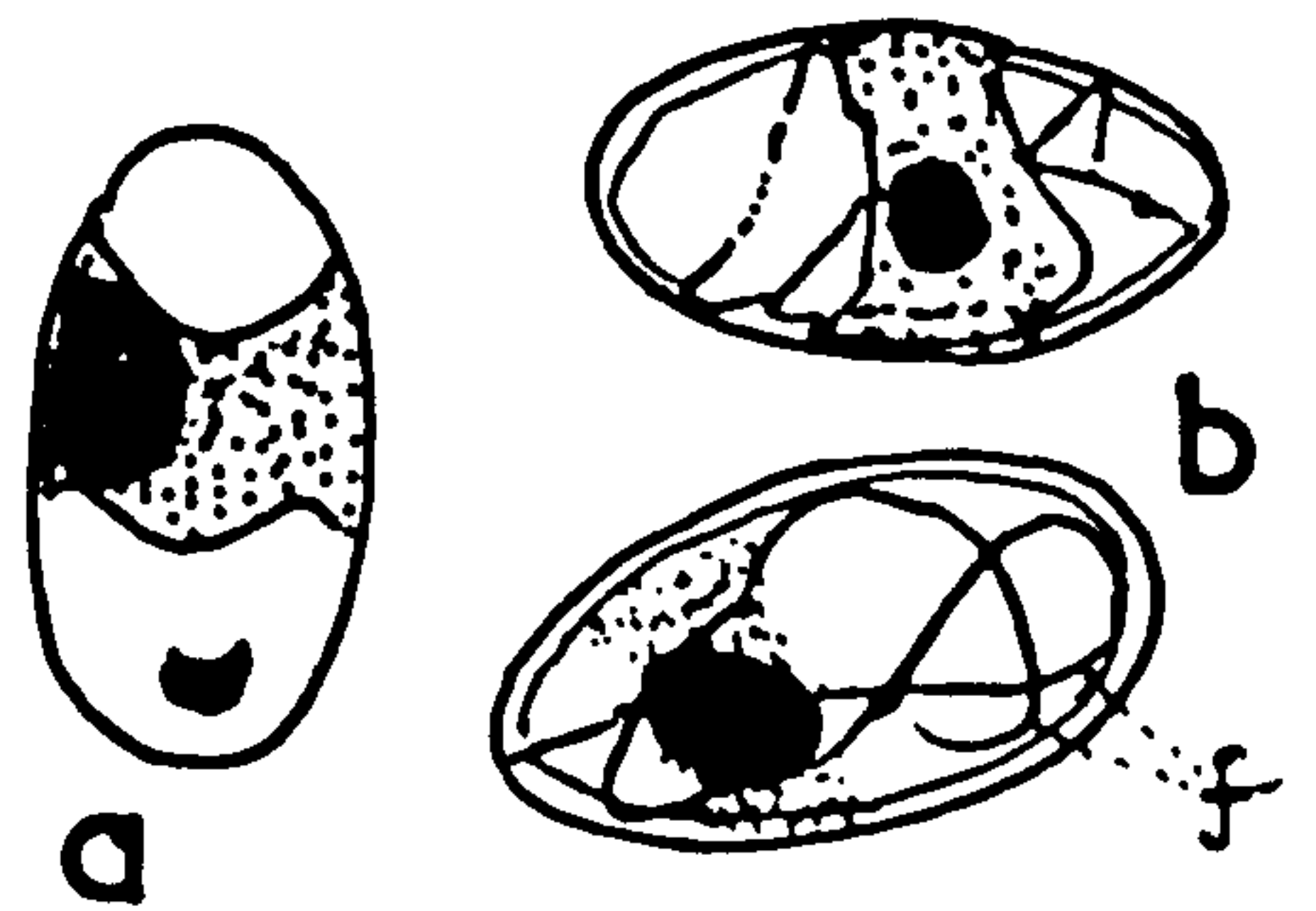
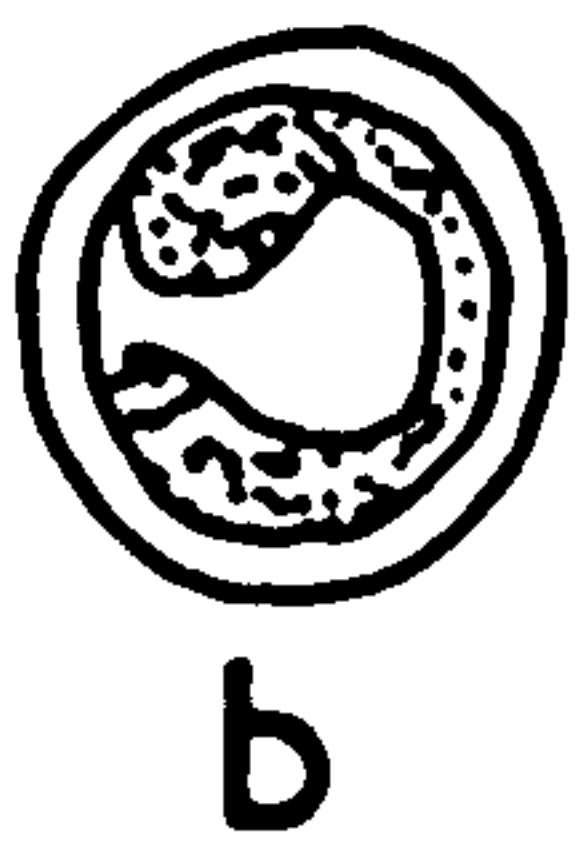
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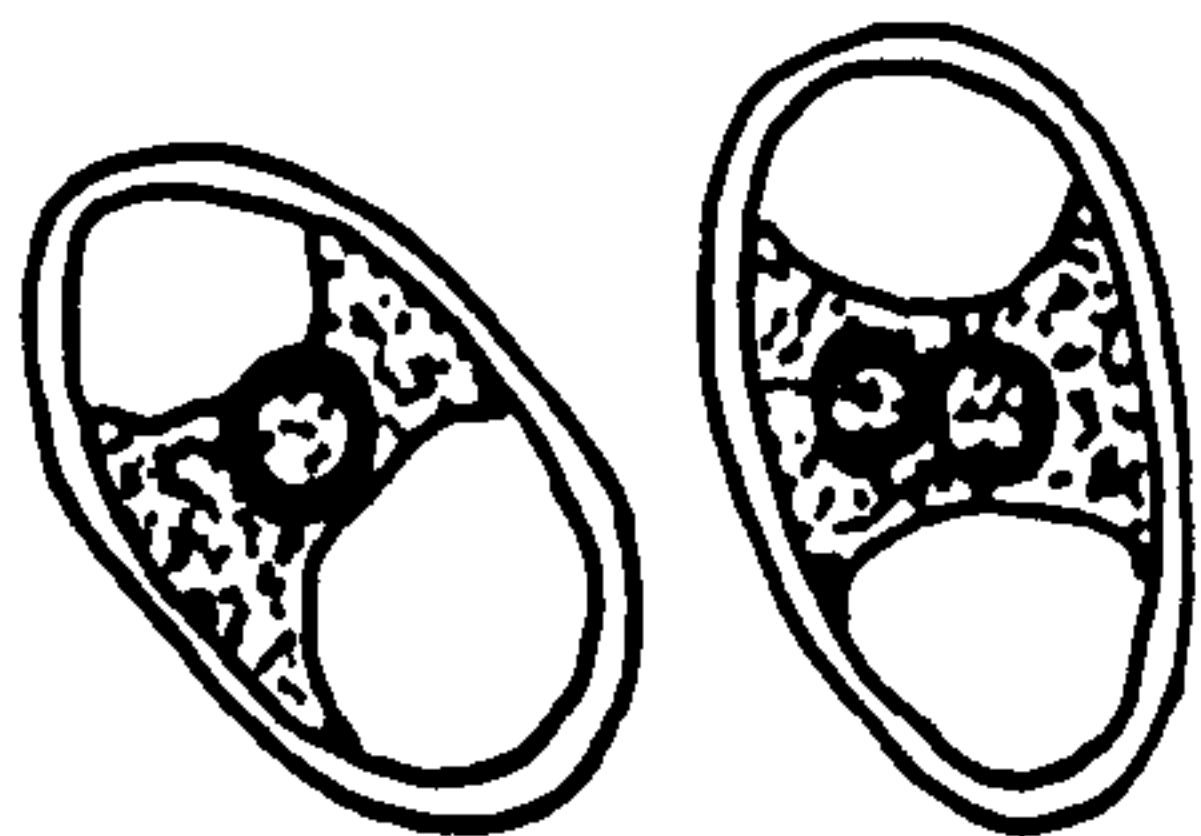
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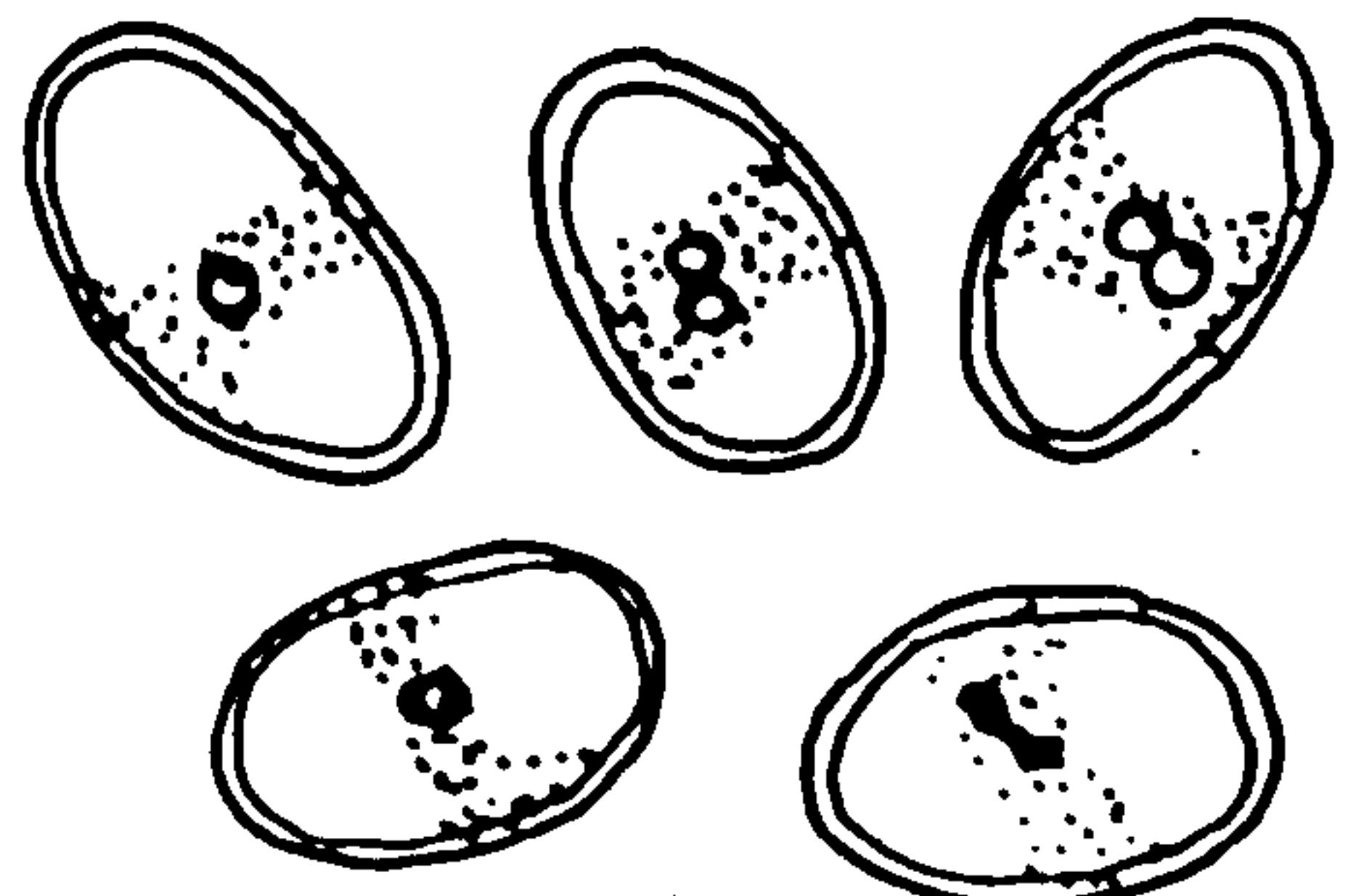
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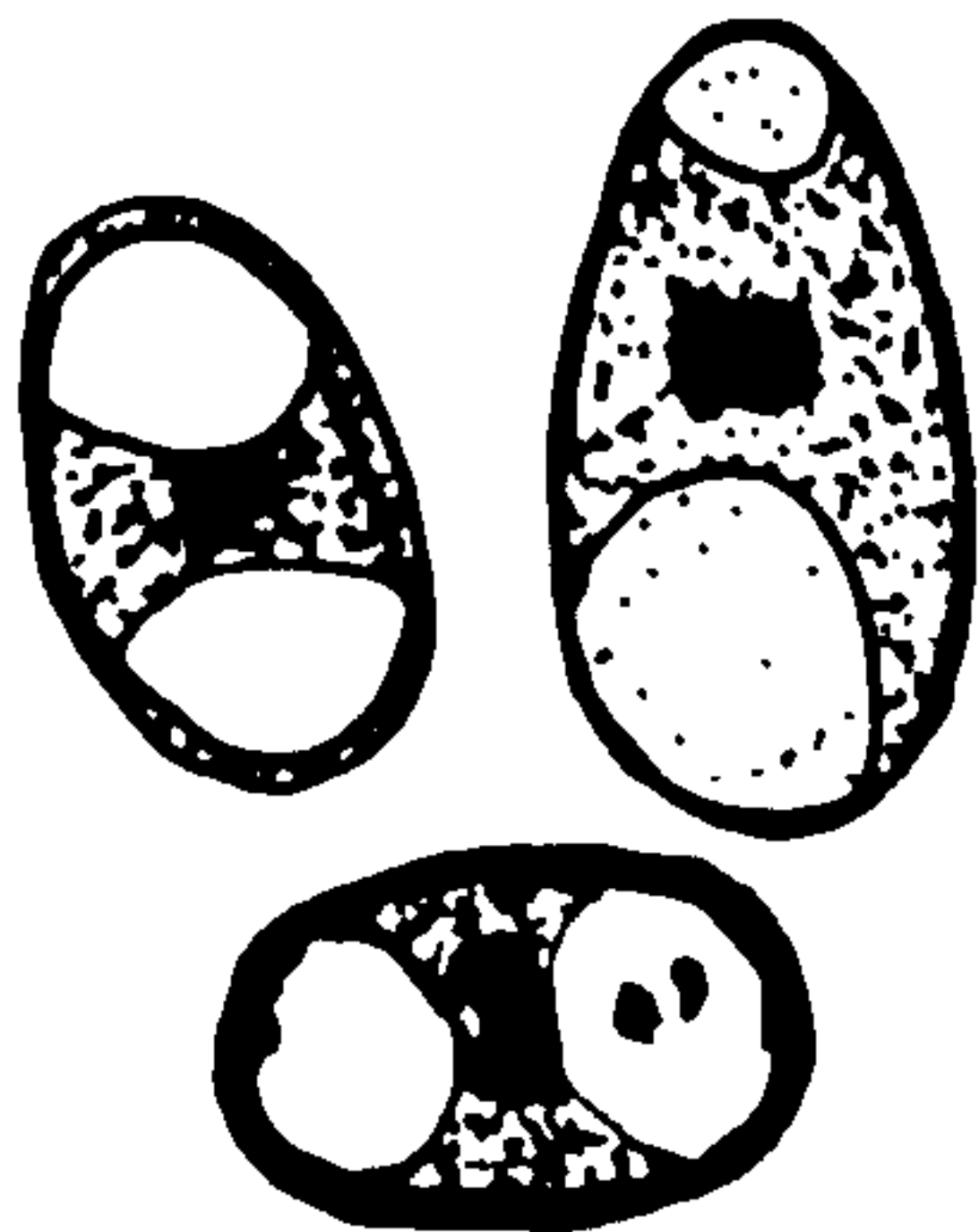
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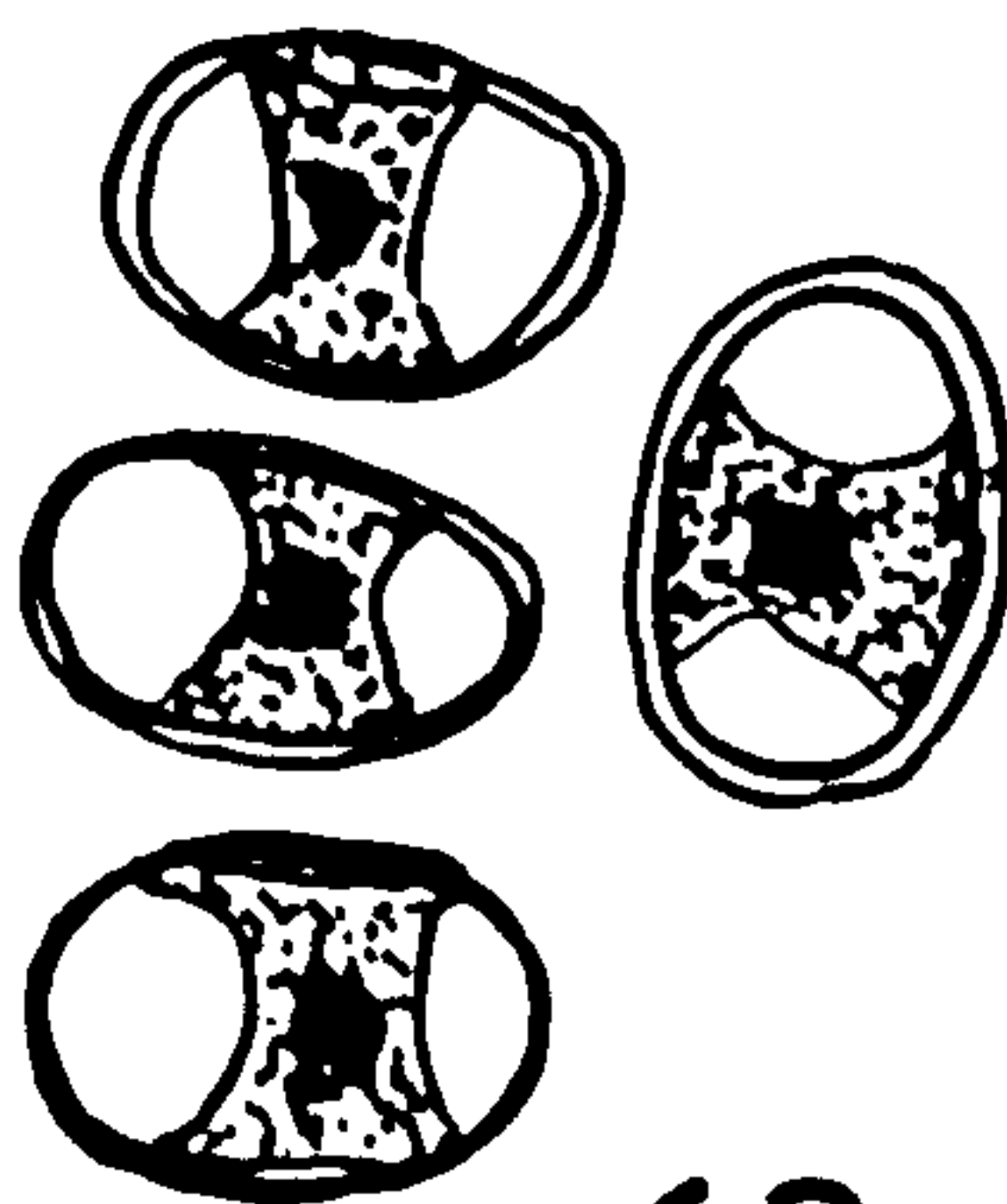
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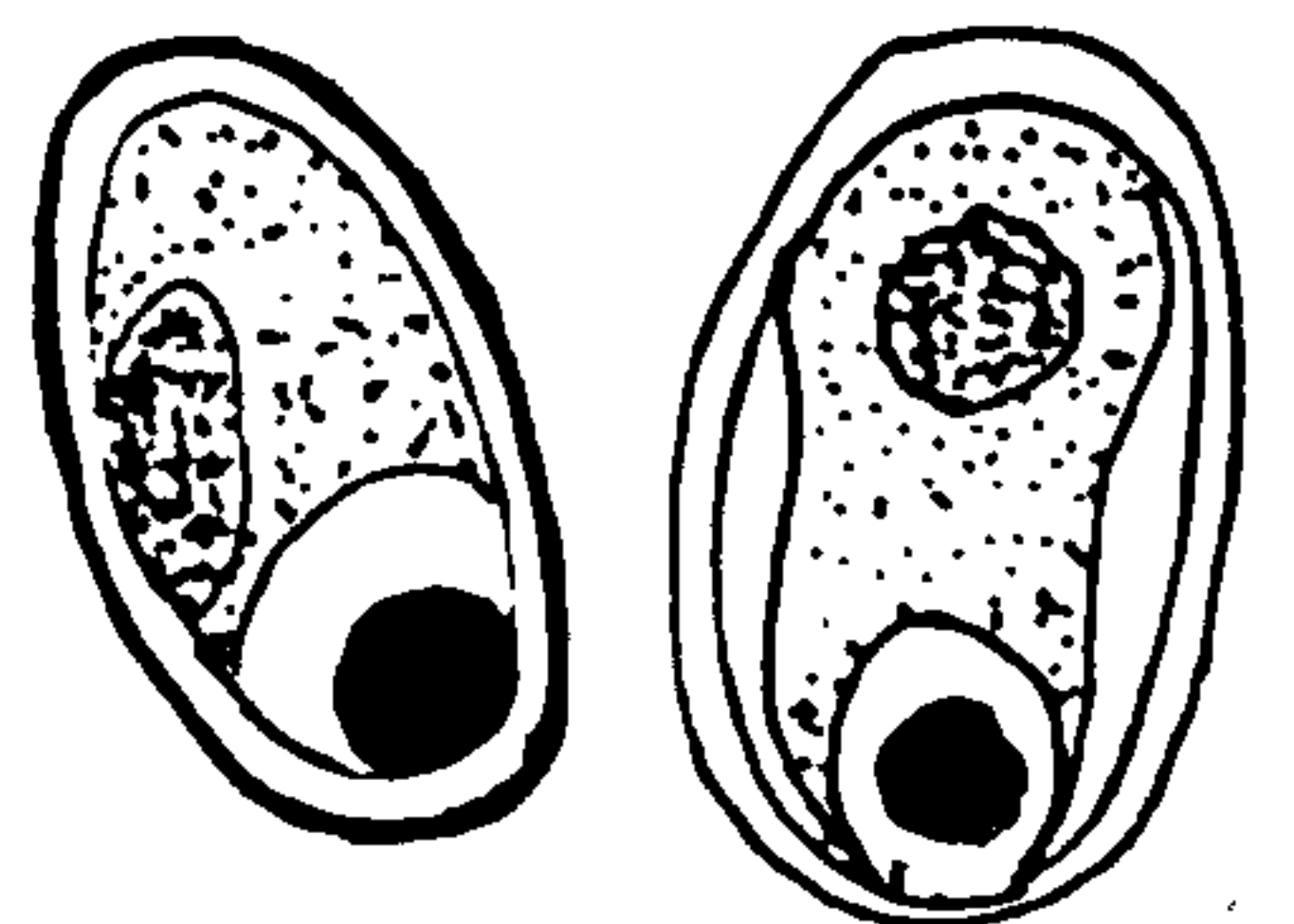
61



62



63



64

10  $\mu$ .

suitable for study of spore structure. In sections stained in Heidenhain's iron haematoxylin there invariably appears at the narrow anterior end of the spore a distinct black granule ( Plate VIII, Fig. 56) from which a thin but distinct filament extends. This filament soon disappears behind the girdle-shaped sporoplasm which is situated at the centre of the spore. The granule is obviously the attachment organelle for the filament and has been observed by many previous workers, for instance in Nosema bombycis (Stempell 1909, KUDO 1916) Plistophora macrospora ( Léger and Hesse 1916 ), P. simulii ( Debaisieux and Gastaldi 1919, Debaisieux 1928), Glugea anomala (Debaisieux 1919) , Plistophora blochmanni (Zwölfer 1926 who called it " Folkorper" ) .

The girdle-shaped sporoplasm which stains very deeply can be recognised as an annular mass in spores that have been sectioned transversely (Plate VIII, Fig.56 b) In over-differentiated sections the nucleus is usually seen as an irregular deeply staining area within this girdle. No other structures are visible in these preparations , but occasionally a black-staining speck in the posterior pole of the spore may represent the metachromatic granule which is seen best in Giemsa-stained preparations, and sections stained by Maximow's method.



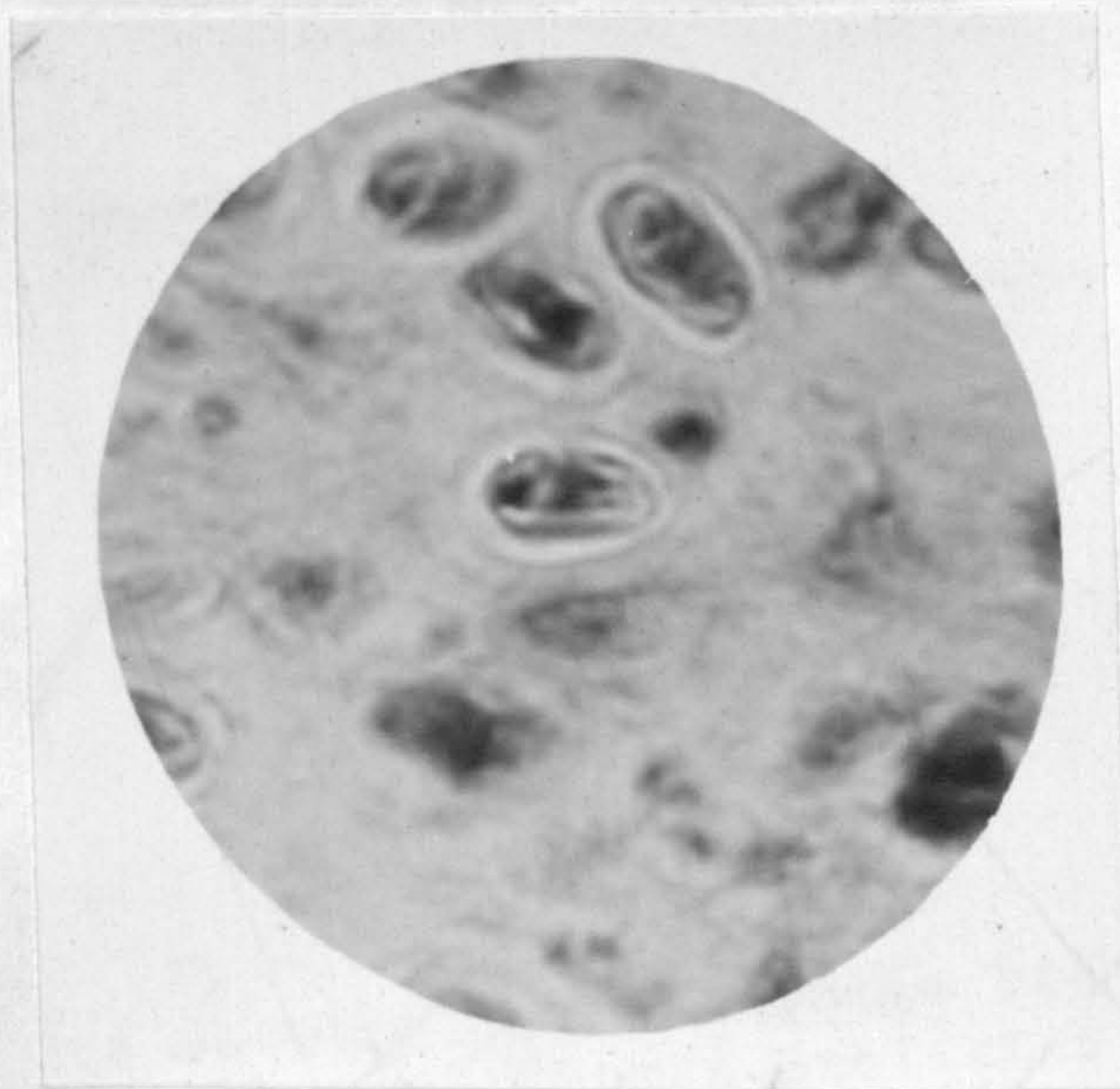


Fig. H

Spore showing metachromatic body in the posterior pole, from a section stained by Maximow's method ( X 2400 )



In sections stained by Maximow's method the posterior vacuole of a large number of the spores shows a bright red body which is either single or made up of several smaller granules. This has been observed in many other microsporidia and it has been called the metachromatic granule (Fig. H) . The significance of this body and its probable chemical nature will be discussed later. Very few other details can be made out in these preparations except the girdle-shaped sporoplasm in which the nucleus stains as a deep blue area (Plate VIII, Fig. 57) Figure 57 c, shows a macrospore measuring 8  $\mu$ . by 3.8  $\mu$ . which was encountered in one of these preparations.

In sections stained by the Giemsa-Colophonium method (Plate VIII, Fig. 58) most spores stain a deep red and the girdle-shaped sporoplasm is found to take on a deep purple stain and shows little differentiation, the nucleus being only occasionally seen (Plate VIII, Fig. 58 a ). In the same preparations that have been over-differentiated, many interesting features can be recognised. In some of these a red-staining granule can be seen at the broader posterior pole with the nucleus occupying a position within a girdle-shaped sporoplasm.



In a few of these , in addition to this a red-staining irregularly coiled thread can be seen (Plate VIII, Fig 59 b) which seems to occupy the whole intrasporal space and is probably the coiled filament which has become disorganised during fixation. That the filament in the above preparations occupies such a wide space is further evidence in favour of the fact that a polar capsule does not exist in these spores. This will be discussed fully when the internal structure of the spore is considered .

In some infected worms, especially those which show the fusiform schizonts, the spores are smaller and sometimes rounder , and in them. the nuclei may appear either single or double; the double nuclei are closely applied to each other (Plate VIII, Fig. 60). In a few worms (Plate VIII, Fig.62) there appear in the same sections spores of both types, and in such cases the normal elongate spores show a single nucleus while the rounder spores have either one or two nuclei (Plate VIII, Fig. 62). Figures 60 and 63 (Plate VIII) show groups of spores of the smaller variety that have been drawn from two different worms which showed fusiform schizonts ( M 125, M 126) . It is clear from the above that the size of the spores is so variable that one cannot demarcate two distinct groups of spores on

size, for there exist all transitions from the longer spores to the shorter ones. All these facts further support the view that the different sized spores occurring in different infections are not spores of different species but merely morphological variations that one could expect due to variations in the process of sporogony. Kudo (1924) claims that these differences "are quite probably due among many unknown circumstances to the environmental conditions in the host body - notably the size nature and condition of the host cell in which the development of the microsporidian took place". Mention may be made of a few species of microsporidia in which such wide variations in size of spores exist - Nosema marionis length 1.5 - 7.0  $\mu$  (Stempell 1919), Stempellia mutabilis length 2.0 - 6.0  $\mu$ . (Léger and Hesse 1910), Stempellia magna length 12.5-16.5  $\mu$  (Kudo 1924). ~~Nosema~~ Nosema binucleatum Weissenberg (1926) says "Die Dimensionen der Sporen variieren nicht unbeträchtlich. So wurden neben Sporen von 6.75 x 3  $\mu$ ., solche 5.25 x 2.6  $\mu$ . und 4.35 x 2.85  $\mu$ . beobachtet".

Finally in sections stained in Feulgen Reagent (Plate VIII, Fig. 61) the nature of the nuclear structure of the spores is verified. In these, all



all appearances depicted in the figures are recognised and it is evident that double nuclei in close contact are those that have delayed to fuse during the earlier phase of autogamy.

### Discussion

In his treatise on sexuality and the chromosome cycle of the Sporozoa, Naville (1931) in summarising the views on the developmental cycles of microsporidia groups them under the following headings -:

1. Interpretations excluding a sexual cycle altogether.
2. Sexuality manifested by a simple autogamous fusion of the two nuclei of the sporoplasm.
3. Fertilization occurring in the interval between schizogony and sporogony. This occurs in three different fashions, by autogamy, isogamy and anisogamy.

He explains the first of these by saying that the stages of fertilization are rare, and that some of the authors who did not recognise such a phase completely ignored certain bi-nucleate stages which were suggestive of a sexual process. For instance in Nosema bombycis Ohmori (1912) describes elongate schizonts containing eight nuclei in groups of two and some of his figures of sporonts clearly suggest a fusion of nuclear pairs.

But this author did not mention any phase of fertilization.

As for autogamous fusion of the two sporoplasm nuclei, Stempell (1909) considered that it took place after the liberation of the sporoplasm from the spore of Nosema bombycis, while Georgevitch (1927 , 1929) believed that in the sporoplasms of Plistophora blochmanni and P. periplanatae the double nuclei represented gametic nuclei. As Naville points out neither of them proved that the fusion of these nuclei took place, but merely took it for granted because the sporoplasm is binuclear while the earliest stages of development are uninuclear. As we have seen above in some spores of N. helminthorum there are two nuclei in apposition while in the majority there is only one. This is merely due to a delay in the completion of the autogamous process that begins during secondary schizogony, or is due to the precocious development of the spore in such cases.

There is a great deal of evidence in many species of microsporidia denoting fertilization between schizogony and sporogony. That this occurs by isogamy was only shown by Mercier (1909) for Thelohania giardi



and by Poisson (1929) for Nosena veliae. The only protagonists of the theory of anisogamy were Guyenot and Naville (1922) who claimed that in Glugea (Plistophora ?) danilewskvi there was a fertilization of anisogametes at the beginning of sporogony. Although they saw what they believed to be rounded macrogametes and falciform microgametes they never observed the actual fertilization. Furthermore Debaisieux (1919) working on the same species did not find any evidence for anisogamy but believed instead that autogamous 'copulae' ( "diplocarya " ) are formed.

The occurrence of an autogamous fusion of nuclei in the interval between schizogony and sporogony has been accepted by the majority of workers. All of them noted that there was a secondary phase of schizogony which culminated in the formation of binucleate schizonts which Debaisieux called "diplocarya", and that by the fusion of the two nuclei a zygote was formed which gave rise to one or several spores according to the genus. For instance Kudo (1924) showed this to be the case in Thelohania legeri and Stempellia magna , Mattes (1928) in Thelohania ephestiae and Debaisieux ( 1919, 1919 a, 1919 b, 1923 ) in a number of microsporidia both monosporous and polysporous. All these workers

agree that there is an autogamy but indicate that it takes place by a variety of methods in different forms. Debaisieux ( 1928) summing up his observations on the mono- and poly- sporous types stated that , in the latter, nuclear fusion always takes place early and the sporont thus formed gives rise to the appropriate number of sporoblasts ( and hence spores), while in the former the nuclear fusion does not take place in the sporont but probably in the spore or in the sporoplasm after liberation.

On examining the above views in the light of the observations made on N. helminthorum , it is clear that autogamous fusion of nuclei occurs either between schizogony and sporogony, or later on, in the spore or sporoplasm after the latter is liberated. All possibilities between the two extremes are found even within the same worm. Thus it appears that in all microsporidia autogamy takes place, but a variety of conditions can alter the time at <sup>which</sup> it occurs. Due to some peculiarity of the parasite or some unknown factor in the internal environment of the host , the fusion of nuclei takes place earlier or later as the case may be. This would explain the fact that in many microsporidia some observers have noted either one or two



nuclei in the mature spore. For instance in Nosema bombycis (Léger and Hesse 1907,) Ohmori 1912 ) Thelohania legeri ( Kudo 1924), Plistophora blochmanni (Zwölfer 1926), Nosema nonagriæ ( Schwarz 1929) and in the present species, either one or two nuclei have been observed. The demonstration by Jirovec (1936), using Feulgen reagent , that many polysporous spores have more than one nucleus, rather invalidates Debaisieux's generalisation regarding these forms.

As to the significance of the process of autogamy which takes place in N. helminthorum , one can only assume that during the division of the second phase schizonts to give rise to binucleate forms (whose nuclei do not separate but later fuse), some chromatin material is given up by the dividing nuclei. This probably leads to reorganisation of nuclear material. Unfortunately no further details of this nuclear division could be made out.

Finally, it is necessary to mention briefly the evidence in support of the fact that fusiform cells are indeed schizonts and not host fibroblasts. In this connection the work of Weissenberg (1926) on N. binucleatum and Kudo (1944) on N. notabilis , leaves no doubt that

the authors met with the same type of cells which they refer to as spindle-form schizonts and which had previously been seen in many other microsporidia by various workers. Both Weissenberg and Kudo describe and picture some of the cells with very long processes not unlike those seen in N. helminthorum. Weissenberg regarded them as aberrant schizonts, but their appearances certainly show that they are far from abnormal at any rate in N. helminthorum. Kudo's demonstration of similar cells in N. notabilis (parasitic in the trophozoites of a myxosporidian Sphaerospora polymorpha) proves beyond doubt that these cells cannot be fibroblasts.

#### The emergence of the Sporoplasm

The mode of emergence of the infective sporoplasm of the microsporidian spore has baffled protozoologists for many years. Kudo (1924) pointed out that "the entire process of emergence of a living amoebula from a spore has unfortunately not been observed in any case". Many views have been expressed on the possible methods of emergence but few have been based on accurate observations. In a brief preliminary communication (Dissanaike 1955) I have suggested the most likely mode of emergence in Nosema helminthorum based on certain



observations I made, and subsequently Canning (1955) has confirmed this for Nosema locustae.

According to the earlier workers extrusion of the polar filament of microsporidian spores is followed by a creeping out of the sporoplasm in amoeboid fashion through the aperture made by the extruded filament. As early as 1888 Pfeiffer claimed that in hanging drop preparations of spores of Nosema bombycis, he saw the sporoplasm emerging, but his diagrams are not at all convincing. Later Sasaki (1897) said that in the blood of the silkworm the sporoplasm of N. bombycis emerged from one of the poles or from the side of the spore. Paillet (1918) made a similar observation on Perezia legeri. Fantham and Porter (1912) described the creeping out of the sporoplasm from the spore of N. apis and they gave details of the subsequent movements of the amoeboid body over the surface of the intestinal epithelium. They did not however, give any evidence for these statements. Many other observers have noted this type of change after extrusion of the filament in various species of microsporidia. For instance in N. bombycis (Stempell 1909, Kudo 1916) N. bombi (Fantham and Porter 1914), N. apis (Trappman 1923) Stempellia magna (Kudo 1925) and Plistophora blochmanni (Zwölfer 1926)

All the above workers believed that the tip of the extruded filament attaches itself to the host epithelial cell and thereby brings the spore closer to the host tissue. According to them the filament then detaches itself and the sporoplasm creeps out through the aperture left by the detached filament. What they all failed to realise was that this orthodox view was quite paradoxical since, as Bailey points out (1954) "the spores would be anchored only to be released when the filament breaks off to allow the planont to emerge". This still leaves the sporoplasm far away from the host epithelium, and it has a long way to travel before reaching these cells.

A more reasonable suggestion was made by Korke (1916) who said that the sporoplasm, attached to the tip of the filament, is probably conducted to the distant part of the tissue by the filament. He found that the binucleate sporoplasm of Nosema pulicis (= N. ctenocephali) was attached to the distal tip of the extruded filament. Since then several workers have observed a protoplasmic globule at the tip of the extruded filament in the species studied by them. Korgenthaler (1922) described a gush of fluid from the distal end of the extruded filament of N. apis but did



not comment on it. Ohshima (1927) first noted in N. bombycis a very viscous fluid at the tip of the filament which he believed adheres to structures with which it comes in contact, and later (1937) showed that the sporoplasm is forced out through the tubular filament and can be seen at the centre of this fluid mass. Trager (1937) and Gibbs (1953) have also seen a protoplasmic globule at the tip of the filament in N. bombycis and Gurleva sp. respectively. Gibbs felt that the filament was hollow and was actually everted like the nematocyst of a coelenterate and that the sporoplasm is thus "injected" into the tissues after the filament has penetrated them. He claims to have seen the filament piercing blood cells in the haemolymph. In fact Ohshima (1937) too regarded that the sporoplasm is injected in this way but he could not accept the hypothesis that the filament is everted, and thought it more likely that it is extruded in a 'jack-in-the-box' manner. As Kudo (1944) remarks these writers failed to take into consideration the fact that the extruded filament is in many instances over 50  $\mu$ . long and has an extremely fine structure with an estimated diameter of 0.1  $\mu$ . They did not give any stages indicating the passage of the sporoplasm through a 'tubular filament', nor did they take into consideration the fact that the

force required to 'inject' the sporoplasm through such a long tubular filament would injure it.

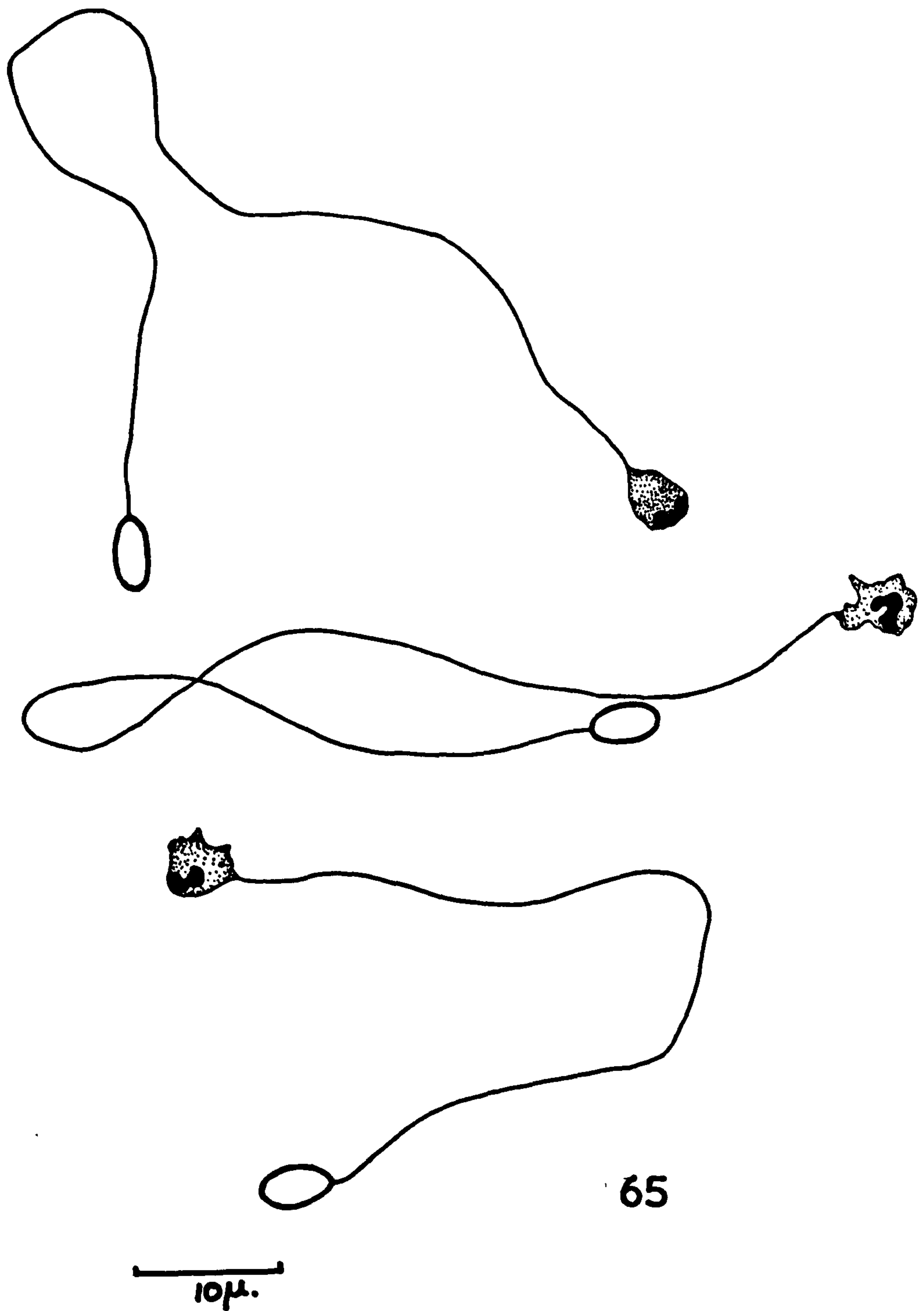
Ohshima (1927) showed that the digestive fluid of the silkworm completely digested the filament in a short time and Zwölfer (1926) showed in Plistophora blochmanni that trypsin completely digests the filaments in 24 hours, an observation similar to which I have made in N.helminthorum. These facts suggest that whatever function is attributed to the filament it must be very momentary and it is most reasonable to accept the recent views that it brings the sporoplasm in some way in close contact with the host tissue, after which it gets digested. Whether the sporoplasm is injected into the tissues as believed by Ohshima and by Gibbs, or whether it is attached to the tip of a non-hollow filament and is thereby 'applied' to the host tissues when the latter is extruded, subsequently working its way into the host tissues by secreting a histolytic substance as I have suggested recently, is left to be confirmed by future workers. But as I shall try to indicate below, the observations I have made on N. helminthorum strongly suggest that the filament is not hollow and that the sporoplasm is normally attached to or is a continuation of the filament, and when the



latter is extruded the sporoplasm must necessarily come out with it. It is easy to explain the failure to see the sporoplasm more often at the tip of the filament that has been extruded by the various artificial methods available. In these instances the process of extrusion is so violent that the sporoplasm is torn away from the filament after extrusion, or is left behind within the spore or at the aperture. It is most probably the last of these possibilities that has led the earlier workers to conclude that the sporoplasm creeps out in amoeboid fashion from the aperture of the spore.

Observations on N.helminthorum - Spores from infected segments of Moniezia expansa left overnight in the refrigerator were examined in a drop of water that was covered with a cover-slip and only lightly pressed. Several spores showed a globular mass of protoplasm at the tips of their extruded filaments. The corresponding spores were completely empty. This preparation was soon dry-fixed after removing the cover-slip gently, and stained in Giemsa stain. The result was a preparation with over twenty spores with extruded filaments showing at their tips irregular spread-out masses of protoplasm which on close examination were seen to be continuations of the filaments. These masses were undoubtedly the sporoplasms and each had a single nucleus also of

PLATE IX



Spores with extruded filaments showing the sporoplasm at the tip of the filament, from a smear preparation of M. expansa(Giemsa)



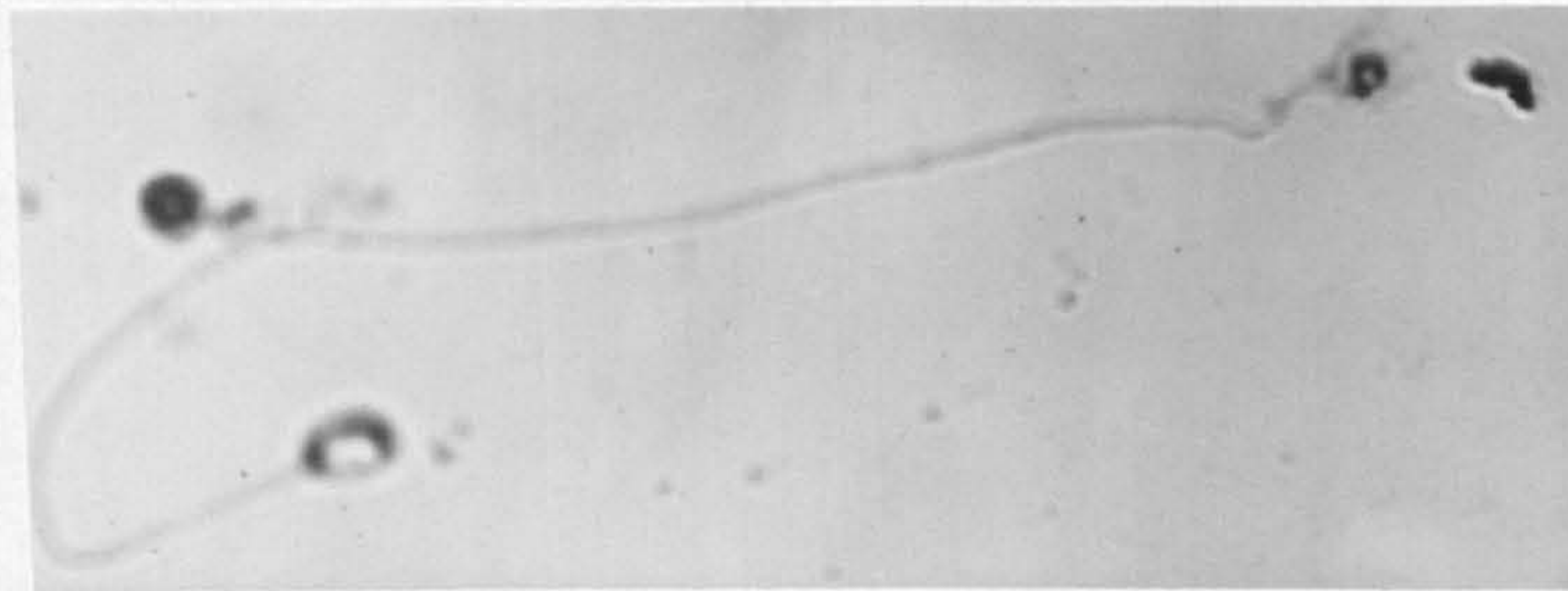


Fig. I.

Spore with extruded filament showing sporoplasm  
at tip of filament from a smear stained in Giemsa  
stain. ( X 1200 )

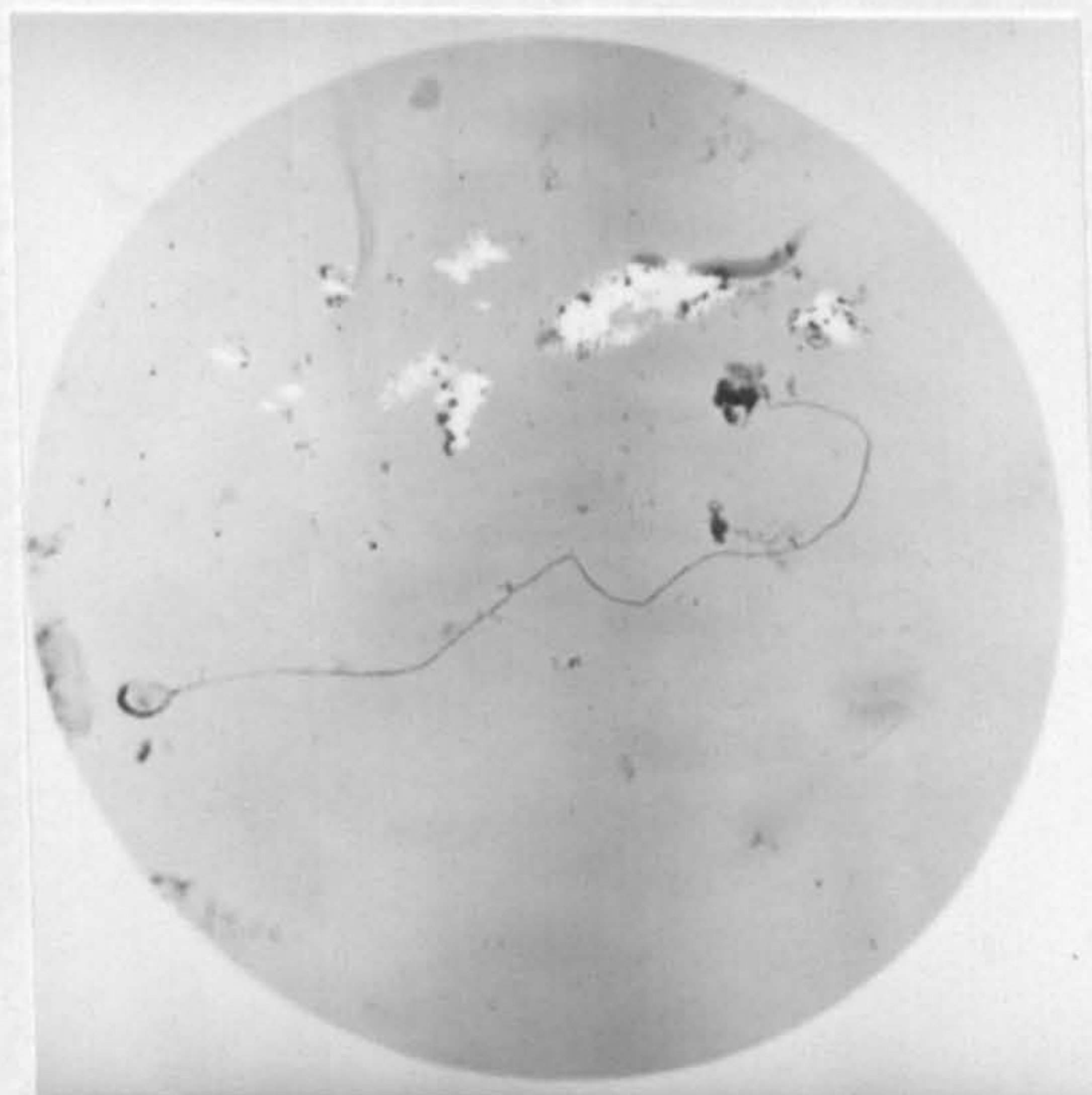


Fig. J.

Same as above ( X 700 )



irregular outline. ( Plate IX , Fig 65 and Figs. I , and J ). Some of the nuclei were dumb-bell shaped (Fig. 65) while others had an annular appearance with a clear space in the middle (Fig. I ).

A similar observation was made on a few spores of N. helminthorum from an experimentally infected Hymenolepis nana of a mouse (Plate XIII, Fig.75 ), and also in one spore of Nosema buckleyi . . . which I found recently in the pupae of a culture of Tribolium castaneum . Here a globular mass of protoplasm was seen at the tip of the extruded filament which though fully extruded appeared short since it was closely coiled .( Part III , Fig. 33 ).

On many previous occasions, spores examined after mechanical extrusion of the filaments showed the following appearances - : In quite a few the entangled filament was seen outside the spore with the distal end still within, while in several spores examined in dilute Toluidine blue spores with extruded filaments were seen with the anterior end of the spore still containing a bluish mass which was probably the sporoplasm.



From all these observations it is concluded that - :

- (a) The sporoplasm is normally attached to the tip of the polar filament, or is a continuation of the filament, which is not hollow.
- (b) When the filament is extruded it does so by merely getting "whipped out" through a weak place in the anterior end of the spore in a typical 'jack-in-the box' fashion.
- (c) The sporoplasm being a continuation of the filament is dragged out with it. In some instances filament extrusion is so violent( especially when this is brought about by artificial methods ) that the sporoplasm gets detached from the tip of the extruded filament or is left behind within the spore or or at its opening. It is evidently this that led the past workers to the conclusion that the sporoplasm creeps out of the spore after filament extrusion.

As the spore is found to be completely empty after the filament is extruded it is very unlikely that a, polar capsule exists , and although the flask-shaped structure described by Thélohan (1894) on treating the spores with 66 % nitric acid was easily demonstrated in N. helminthorum , ( Plate VII, Fig. 55 ), I am of the

opinion that this is really an artifact produced by the contraction of structures such as the sporoplasm and the filament, which being attached to the anterior end of the spore, give the impression that this so called polar capsule is situated at the anterior  $2/3$  of the spore.

My views on the mode of emergence of the sporoplasm have been confirmed by Canning (1955) who found that the sporoplasm of N. locustae is similarly attached to the tip of the filament. She too was of the opinion that the filament is not hollow and does not penetrate the host tissue, and also that a polar capsule does not exist in that species. More recently Krieg (1955) by Electron microscope studies of the filament of Plasmodium melolanthae showed that it is a solid structure which is  $0.1 \mu$ . thick and he concluded "Der in der Polkapsel spiralig aufgerollte Faden wird bei deren Vakuolyse (infolge erhöhten osmotischen Druckes)..... durch den ausschliessenden Vakuoleninhalt mitgerissen."

The above findings have led me to a newer conception of the internal arrangement of the structures within the spores of the microsporidia belonging to the Nosematidae which will be discussed in the next section.



Internal Structure of the Spore

There is a great deal of uncertainty regarding the finer structure of the microsporidian spore and much diversity of opinion prevails on the matter. This, as Kudo (1924) points out, is "doubtlessly due to the smallness of the object, to the peculiar nature of the spore membrane which obscures the internal structure and to the dissimilarity in structure in different species".

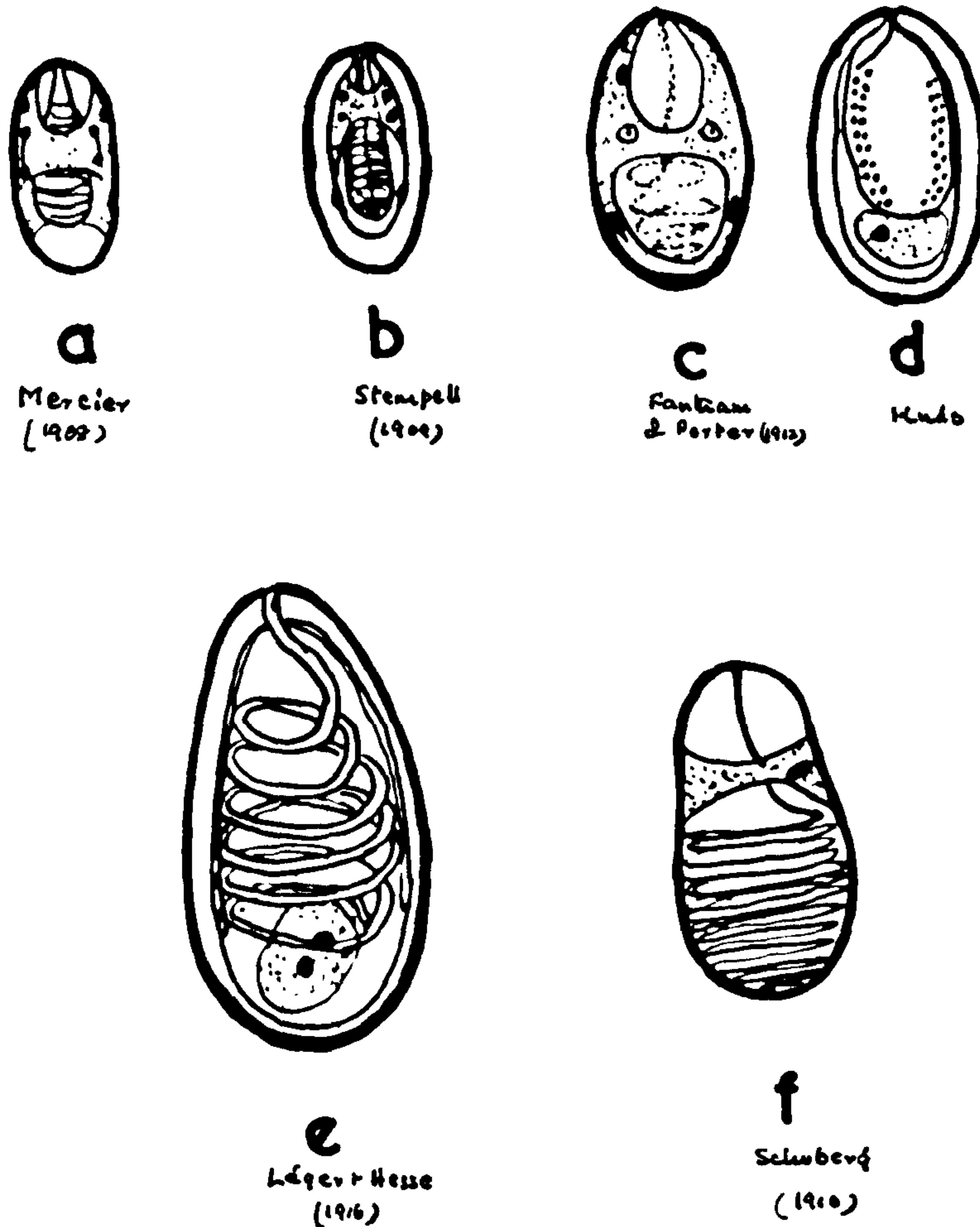
The fresh spores of N. helminthorum, and in fact of most other microsporidia belonging to the Nosematidae, are highly refractile, and apart from the posteriorly placed vacuole show no other details of structure, even under the phase contrast microscope. It has therefore been necessary to base our ideas of the internal structure of these spores on the results of fixation and staining. This has led to a great deal of difference in interpretation by different workers, as has been evident in the case of the same species studied by different workers. Furthermore as Jirovec (1936) has shown in the case of Heidenhain's haematoxylin, a number of artifacts can occur by these staining methods which must be carefully considered. My observations on

the spores that have extruded their filaments and the demonstration of the fact that the sporoplasm is attached to the tip of the extruded filament have led me to an idea of the internal arrangement which I believe holds for nearly all the microsporidia belonging to this family. But before going into this it is necessary to mention very briefly the main views of past workers.

It was Thélohan (1894) who upon treating the spores with 66 % nitric acid at 38°C, demonstrated what he believed to be the polar capsule in the microsporidian spore. This he showed to be at the end opposite the vacuolar end. He regarded this polar capsule as normally invisible due to the coat of protoplasm which surrounds it. He believed that the sporoplasm contained three nuclei one of which was the nucleus of the polar capsule and the other two those of the sporoplasm proper. Stempell (1904) first gave a diagram representing his idea of the spore structure of Glugea anomala and modified it later in 1909 for Nosema bombycis (Text Fig. b). According to him, within the spore shell are a small and a large vacuole situated respectively at the anterior and the posterior ends. The sporoplasm he considered to be girdle-like and lying between the two vacuoles, and containing four nuclei when the spore is mature. Stempell



showed that the polar filament begins at or to one side of the anterior tip of the spore and coils back through the sporoplasm to the posterior vacuole where the major part of the filament is situated. At first he did not



mention or depict a polar capsule but in 1909 he figured a polar capsule with its nucleus, and also two parietal nuclei for the spore membrane. Similar views were held for Thelohania giardi by Mercier (1903) (Text Fig. a ), and for T. chaetogastria by Schroder (1909). On the

other hand Schuberg (1910) working on Plistophora longifilis considered the filament to be directly coiled under the spore membrane, the sporoplasm to contain only a single nucleus and the polar capsule to be non-existent (Text Fig, e) He claimed that the nuclei seen by other workers are none other than the volutin or metachromatic granules which appear in the spore by fixation and staining. Weissenberg (1913) agreed with this for G. anomala and G. hertwigi.

Fantham and Porter (1912 , 1914) gave the structure of the spore of N. apis and N. bombi as follows ( Text Fig. c ). They distinguished two nuclei for the sporoplasm, two for the spore membrane and one for the polar capsule which apparently they regarded as corresponding to the anterior vacuole. According to them the filament passes through the capsule and the sporoplasm as a straight rod and then becomes coiled in the posterior vacuole.

Kudo (1916) held a similar view to that of Mercier and Stempel except that he observed only two nuclei for the sporoplasm.

Léger and Hesse (1916) in Plistophora macrospora considered the polar capsule to lie close to the shell and to contain a filament that was spirally coiled six



times . They noticed the sporoplasm as a rounded binucleate body situated in the posterior vacuole. According to them the girdle-shaped structure seen by other workers was the contracted substance containing the capsule and the many nuclei seen in this were , they thought, the turns of the filament in optical section (Text Fig. e).

Georgevitch (1917) agreed with the views of Leger and Hesse for N. marionis . Paillot (1918) for Perezia mesnili accepted the views of Fantham and Porter . Debaisieux (1919 ) agreed with Schuberg's view for the structure of Thelohanina varians .

For a larger species of microsporidian, Stempellia magna , Kudo (1925 ) found a binucleate sporoplasm at the posterior third and a conspicuous polar capsule at the anterior third. Finally in N. apis ( Text Fig. d ) the same author found a similar structure with " a uninucleate sporoplasm, and the filament coiled fifteen times along the polar capsule " inside of which and continuous to it is coiled back again toward the tip where the filament is attached ."

Among subsequent workers mention must be made of Zwölfer (1926) who did not think a polar capsule

existed in Plistophora blochmanni and regarded the sporoplasm to be the girdle-shaped structure with one nucleus. He also, like Schuberg and others, observed metachromatic staining structures in the posterior vacuole ; Hesse (1935) in Pyrotheca incurvata found the sporoplasm in the posterior position.

It is seen from this short review of the literature on the spore structure of the Nosematidae that there are two main views: -

- (1) The spore is pyriform or ovoidal with a polar capsule at the anterior half or two thirds of the intrasporal space; the sporoplasm is at the posterior pole.
- (2) The spore is ovoidal or ellipsoidal ; the sporoplasm is a girdle-like ring located at the middle of the spore and surrounding the spirally coiled filament which may or may not be within a polar capsule.

My studies on N. helminthorum convince me that the arrangement of structures is as suggested in the second view. I do not, however, think that a polar capsule exists for many reasons :-

- (a) The spore is completely empty after the



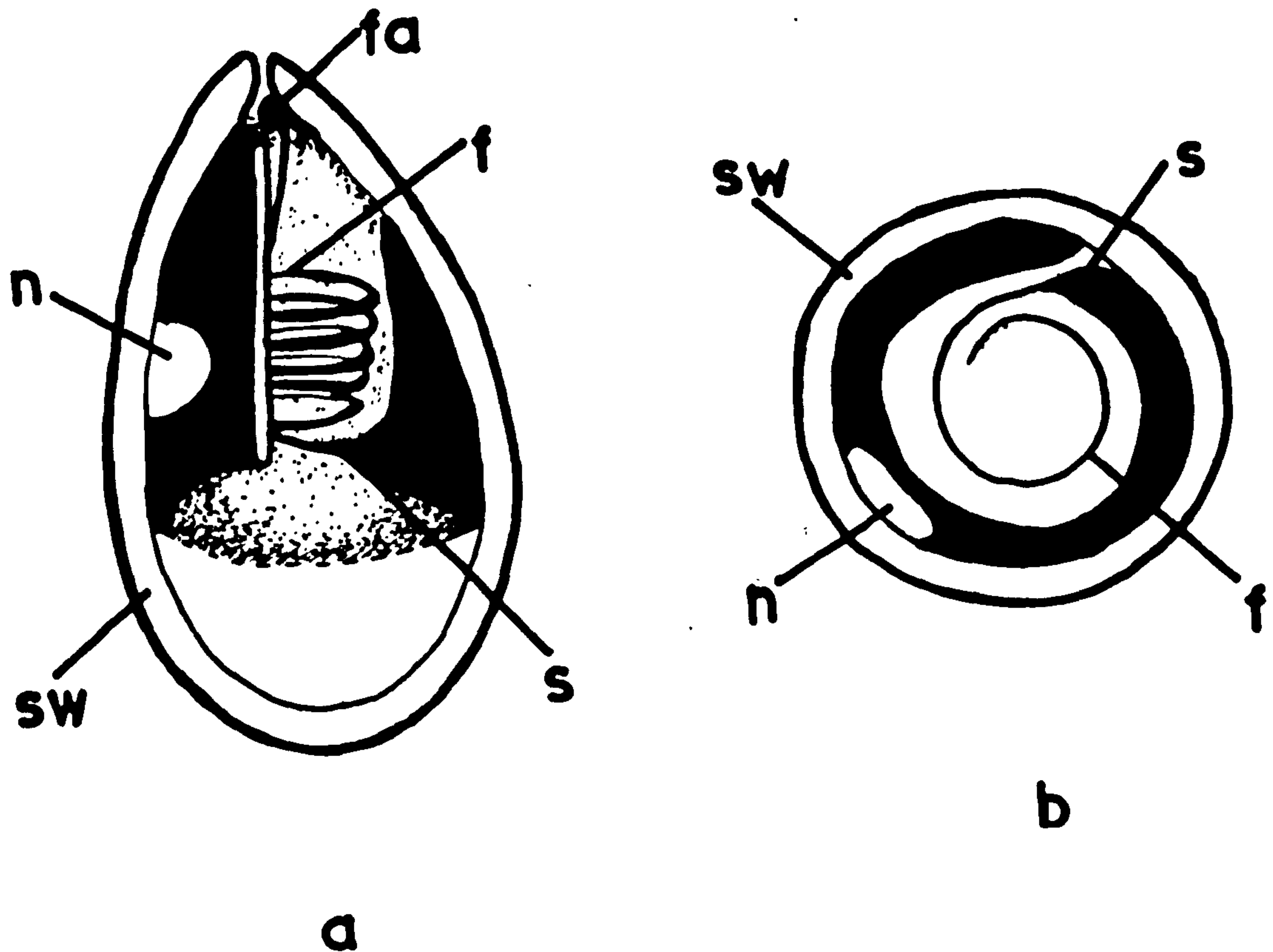
filament is extruded .

- (b) The appearance of a pear-shaped polar capsule in spores after nitric acid treatment is merely an artifact.
  - (c) If the sporoplasm is situated outside the polar capsule as many workers believe, then it would still be imprisoned in a compartment shut off by the spore wall on the one hand and the polar capsule on the other, unless one imagines there is another exit for the sporoplasm.
  - (d) The appearance in certain Giemsa stained sections suggests that the filament occupies the whole intrasporal space ( Plate VIII, Fig. 59 b).
  - (e) And finally as shown for N. helminthorum in the present work , and for N. locustae (Canning 1955), the sporoplasm is either a continuation of the free end of the filament or is attached to it. This proves that if a polar capsule exists at all it must include the sporoplasm within it . But this cannot be accepted owing to the appearances in stained preparations.
- It is therefore concluded that the filament

is spirally coiled within the central axis of the spore and that it is continuous at one point with a very 'fluid' sporoplasm which surrounds it in a girdle-like fashion . This sporoplasm is so closely adherent to the inner surface of the spore wall that it makes the fresh spore appear very refractile, and in those species where it is not large enough to cover the whole inner surface of the spore wall a vacuolar appearance is seen in the posterior pole . On fixation and staining the sporoplasm shrinks considerably from the two poles and so appears as a girdle at the s centre. The filament is attached to the anterior tip of the spore by a thickened granule which has been called the "Polkörper" by Zwölfer (1926) and "Corp polaire " by Debaisieux (1928). When the filament is extruded through a weak spot at the anterior end of the spore , the sporoplasm is dragged out with it for the simple reason that it is attached to its tip.

The diagram overleaf shows what I believe to be the arrangement of structures within the spore of N. helminthorum and perhaps holds true for most of microsporidia belonging to the Nosematidae ( Text Fig. 111)





Text Fig. iii.

Diagrammatic representation of the structure of the spore of Nosema helminthorum L.S. (a) & T.S(b)

- fa - Filament attachment
- f - Filament
- n - Nucleus of sporoplasm
- s - Sporoplasm
- sw - Spore wall

## Chapter IV

### Some Chemical and Cytochemical considerations

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#### The Chemical nature of the Spore wall

Although the spore wall of the microsporidian spore has for a long time been known to be resistant to various physical and chemical agents little is known about the chemical nature of this membrane. Thélohan (1895) pointed out that it is not of cellulose. Auerbach (1910) said " Die chemische Zusammensetzung der Schale ist meines Wissens noch nicht sicher bekannt ". It was only in 1921 that Kudo working on the spores of Nosema bombycia and N. apis concluded that " the spore membrane behaves very much like chitin under the influence of mineral acids ". Koehler (1921) by using a modified van Wisselingh test stated " Es dürfte demnach erweisen<sup>sein</sup> dass<sup>die</sup> Sporenschale der Nosema apis aus Chitin besteht ". However Kudo in his monograph on the Microsporidia (1924) still continued to regard the chemical nature as not definitely known, and Richards(1951) in his "Integument of Arthropods" says "although the evidence for the presence of chitin in the microsporidian spore is not convincing it is logical



to assume it is - due to the habitat of the spores ". In views of Koehler's work it seems surprising that such doubt still exists on this problem , and so an attempt has been made to establish definitely the structure of the spore wall in Nosema helminthorum.

The first tests that were carried out were histochemical, on tissue sections that were fixed in Carnoy's Fluid. Both the Zander and the Bethe tests for chitin were performed after softening the sections over night in Diaphanol, but these tests gave negative results even with control sections containing arthropod cuticle. It was then decided to try out a modification of the van Wisselingh's test. As mentioned earlier the Campbell modification (1929) was used making suitable adjustments for the material used. A characteristic rose-red or purple colour was obtained in all the spores by this method. A piece of cockroach cuticle treated similarly gave the same colour changes. There was no doubt, therefore that the coloration was due to the formation of Chitosan sulphate, and that the spore wall was composed of chitin.

The spores thus coloured dissolved only partially in 3 % acetic acid.

The spores after the chitosan colour test

appeared rounder , and being now empty showed the thick wall more clearly. At the anterior end of the spore a truncated appearance was observed where the wall was apparently dissolved off by the violent treatment with alkali.

### Discussion

The results of the present investigation leave no doubt as to the chemical nature of the spore wall of N. helminthorum. Kudo(1921) and Koehler(1921) believed the same to be true of the spores of N. bombycis and N. apis . Canning (1955) after performing a similar experiment as the above on the spores of N. locustae , showed that a positive chitosean test was obtained . Thus it appears that all microsporidian spores possess a wall of chitin and future tests on the numerous other species are necessary to confirm this. Koehler wondered whether the spore wall of the microsporidia of fishes too consists of chitin. As the species he worked on was a parasite of an arthropod he probably felt that the influence of the surrounding medium was important. It is however only reasonable to assume that structures of organisms of the same order must necessarily be of the same composition, although Richards (1951) suggested that spores in an arthropod habitat could be expected to have a chitinous structure. The



present demonstration that the spore wall of a microsporidian from a cestode consists of chitin, suggests that all microsporidian spores possess a similar chemical structure irrespective of whether the host is an arthropod or not.

### The nature of the Metachromatic bodies

The metachromatic bodies occurring in N. helminthorum and which were mentioned earlier in this thesis were first observed in this microsporidian by Weiser (1951). They are present in a number of other microsporidia although in many others they have not been seen. Their presence in the present species as a definite structure in many of the spores, and their absence in others, suggests that they are probably used up in some way during the maturation of the spore. As indicated before, these are represented in the sporont stage by the 'pre-metachromatic granules' which are definite structures present in the sporonts. They appear in the sporonts as distinct granules lying in a clear space or vacuole. They appear to be extra-cytoplasmic in position. As it was likely that a knowledge of the chemical nature of these metachromatic bodies might shed some light on their probable role,

and help further in the understanding of the organisation of the spore, it was decided to try a number of histochemical tests on them.

The phenomenon of metachromasia is a very complex one and a large number of substances are known to stain metachromatically with some dyes like Toluidine blue, Thionin and the Azures. The physico-chemical nature of metachromasia is not completely elucidated as Gomori(1952) points out, but it may be stated that as a rule certain highly polymerised substances give rise to this effect.

As it was not possible to make a thorough study of these bodies myself I consulted Dr. A.G. Everson Pearse of Post Graduate Medical School, who suggested the most suitable tests to be carried out, and who himself kindly performed some of these tests for me. He suggested the following tests :- The Unna-Pappenheim stain (for DNA and RNA) the Feulgen reaction(for DNA), the Hotchkiss Periodic Acid Schiff test( for mucins, mucopolysaccharides, glycogen, mucoproteins and various lipid components ), and the Sudan Black stain for lipids. These tests were carried out on sections of infected worms using control slides in each case. In addition the Best's test for glycogen was performed, and the Hotchkiss test repeated using



Hale's (1953) modification. All these tests gave negative results .

After carrying out certain tests himself Dr. Pearse informed me that he obtained a positive reaction with the Unna-Pappenheim stain where these bodies showed an affinity for Methyl green. He also found that these bodies contain no phospho-lipid of any kind. He was then of the opinion that they consist of a highly polymerised Desoxy-ribonucleic acid or a Desoxy-ribonucleic acid-protein, and believed that the negative Feulgen reaction that I obtained was not readily explicable unless the fixative used was Bouin's Fluid. But since I had in fact used Carnoy's Fluid for the material on which the Feulgen reaction was used, this explanation was not satisfactory. Since then he has been unable to go further into this matter owing to pressure of work, but informs me that " the bodies, if they are not composed of DNA, must be some kind of phosphatide- or sulphatide-containing lipid", and that extraction techniques might be helpful. Unfortunately lack of time makes it impossible to go into this matter any further, and so the problem of the chemical nature of these bodies must remain unsolved.

In conclusion it is interesting to note the views of past workers on this question. Schuberg (1910) was the first to observe these bodies in Plistophora longifilis and he called them 'metachromatic granules'. He found them staining red with Toluidine blue and Polychrome methylene blue, and dark greyish-brown with Thionin. He surmised that they were some mucoid material which normally fills the whole intrasporal space, and which clumps together under the influence of fixatives. He also stated that they probably swell under certain conditions and thereby force the filament out. These bodies were also seen by Weissenberg (1913) in Glugea anomala and G. hertwigi, where he described them as large round volutin grains in the posterior vacuole. Zwölfer (1926) observed them in the spores of Plistophora blochmanni. He found that these bodies in immature spores broke up into smaller granules as the spores matured and finally disappeared. He showed that in spores left in tap water for a fortnight, these granules were absent, and hence concluded that they had something to do with the maturing of the spore. Jirovec (1936), after studying the spores of a number of microsporidia by Feulgen staining, stated that these bodies were mistaken for nuclei by many earlier workers and that there was no trace of any chromatin material in



them, but he was unable to say what their chemical nature was. There is no doubt therefore that a thorough study of these bodies will be of great value in future work on the microsporidia.

## Chapter V

### 1. Life-Cycle of Moniezia and the role of Oribatid mites.

Until the classical work of Stunkard (1937-1941) the life cycles of Moniezia and other anoplocephalid cestodes remained obscure despite the intensive studies of a large number of workers. Stunkard showed that certain free-living soil mites belonging to the Oribatidae, were vectors of Moniezia, Cittotaenia and Bertiella. Since then a large number of these mites have been incriminated as intermediate hosts of many genera of anoplocephalid cestodes. It is believed that all cestodes of this family are transmitted by these mites which are not uncommon on pastureland and among collections of leaves.

The life cycle of Moniezia is briefly as follows :- The eggs are passed in the faeces of sheep, most of them still within gravid segments. In the soil, the segments disintegrate and the liberated eggs are eaten by the appropriate mites. After the eggs reach the intestine of the mite, the oncospheres work their way into the haemocoel with the aid of their hooks. In the haemocoel, rapid multiplication of the germinal cells of the larva takes place, and in a few weeks a spherical



immobile stage is formed with the hooks now functionless. At about the eighth week the larva is pear-shaped, and calcareous bodies develop in the parenchyma. By the tenth to the twelfth week it has a tail-like cercomere which carries the functionless hooks. At this stage the rudiments of suckers may be seen. A few weeks later the typical cysticeroid larva is formed which has a spherical body and suckers. The cercomere now becomes a fibrous appendage. This larva is now infective and when the mite is swallowed by a susceptible lamb, the larva escapes in the intestine of the new host and develops there, reaching sexual maturity in about three months.

In G. Britain Rayski (1945, 1947) has shown that under experimental conditions a large number of species of oribatid mite can act as vectors of Moniezia, although he found that in Scotland the only natural vector was Scutovertex minutus. He explained this as due to the feeding habits of this mite which bring it into close contact with the eggs of these cestodes present in the faeces of infected sheep.

## 2. Experiments with Oribatid mites

As oribatid mites are known to be the vectors

of Moniezia, it was natural to expect that these arthropods might play some part in the transmission of N. helminthorum from one Moniezia to another. This appeared more likely because arthropods are the usual hosts of the microsporidia, and it was quite possible that an infected mite could pass on the infection to the larval stage of this worm. Accordingly, the literature was carefully examined in order to see what protozoa had been reported from oribatid mites, and whether any microsporidia had been found in them.

Nicolet (1855) first reported and figured two gregarines from Damaeus and other oribatid mites, and named one of these Gregarina oribatium, although he referred to them as helminths ! Michael (1881) in his "British Oribatidae" says "many parts of the canal but more especially the ventriculus, contain gregarines as internal parasites ". Wellmer (1911) mentions Gregarina sp. as occurring in Oribata geniculata but gives no further details. Describing single celled parasites of the Ichthyosporidium type, Thor (1930) mentions a few of these forms under various species of oribatids. Finally, Warren (1944) describes a new haplosporidian which he names Acoccidium ventriculi,



from the ventriculus and the two large lateral caeca of an oribatid mite Hermannia sp. The appearance of this parasite and the dimensions of its spores show that it is definitely not microsporidian.\*

It is thus seen that no microsporidia have been recorded from these mites, but it must be remembered that very few workers have studied the parasites of these mites and it was only recently, after their importance as vectors of anoplocephalid cestodes was discovered, that these mites came to be studied in any detail. It was therefore decided to examine large numbers of these mites from different localities to see if any naturally occurring microsporidia could be found in them. Mites obtained from Kent, Oxford and St. Albans, were examined by either squashing them in a drop of saline, or by sectioning after double-embedding. The results of these examinations are given in the table below. A large number of mites belonging to several of the genera examined harboured cephaline gregarines in their midguts and caeca. There was no trace of microsporidian infection in any of the 338 mites studied in this way. Many of the mites were sectioned in series and so it was

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\*I am grateful to Dr. G.O.Evans and Mr. E. Browning of the British Museum(Natural History) for allowing me to examine the original type slides of Acoccidium ventriculi.

not possible to examine a larger number.

Date	Oribatid	No.	Observation	Locality
29.1.54	<u>Euzotes globula</u>	3	Gregarines in 2)	Oxford
	<u>Xenillus tegeocranus</u>	5	" in 2)	
1.2. 54	<u>Ceratoppia bipilis</u>	5	" in 2)	}
	<u>Scheloribates</u> sp.	1	- ve	
11.2.54	<u>Ceratoppia bipilis</u>	5	Gregarines in all-	Oxford
	<u>X. tegeocranus</u>	10	" in 3)	}
	<u>X. tegeocranus</u>	5	- ve	
17.5.54	"Notaspids"	5	- ve	}
	<u>X. tegeocranus</u>	4	- ve	
18.5.54	<u>Punctoribates puncta</u>	7	Gregarines in 2 ) several in syzygy)	}
21.5.54	"Notaspids"	2	- ve	
	<u>Felops</u> sp.	3	- ve	}
	<u>Punctoribates puncta</u>	12	Gregarines in 3 )	
25.5.54	<u>E. globula</u>	10	" in 3)	}
	<u>E. globula</u>	2	" in 1)	
	<u>X. tegeocranus</u>	3	- ve	
	<u>X. tegeocranus</u>	2	- ve	
	<u>C. bipilis</u>	5	Gregarines in 3)	
23.5.54	"Notaspids"	4	" in 1 -	Kent
29.5.54	<u>C. bipilis</u>	1	- ve	- Oxford
31.5.54	"Notaspids"	8	-ve	- St.Albans
1.6.54	"Notaspids"	5	- ve	- Kent
3.6.54	<u>X. tegeocranus</u>	1	- ve	}
	<u>Belba</u> sp.	2	Gregarines in 1)	

Lites fed with eggs of Moniezia expansa-

X. tegeocranus 58 Gregarines in many,  
Assorted ("Notaspids" 95) - 165 "



From the results of this study it was thought improbable that these mites passed on the microsporidian to the larval stage of the tapeworm. It was more likely that the mites acted as mechanical transmitters of the spores swallowed by them. In this way the spores could be protected from the gastric juices of the sheep until they reached the site where the adult worms were already present. However it was considered interesting to see whether

(a) Oribatid mites could be infected experimentally with this microsporidian.

(b) And if this was possible, in a large number of cases, whether mites experimentally infected with Moniezia larvae could subsequently be infected with the microsporidian or 'vice versa', in order to see if the infection could be passed on to the larval stage of the cestode from an experimentally infected mite.

For these experiments it was found necessary first to select the species of mite that occurred most frequently in the collections. It turned out that Xenillus tereocranus and some other smaller mites with pointed pteromorphs labelled "Notaspids" (as detailed identification was not practicable), were present in largest numbers. As X. tereocranus was a

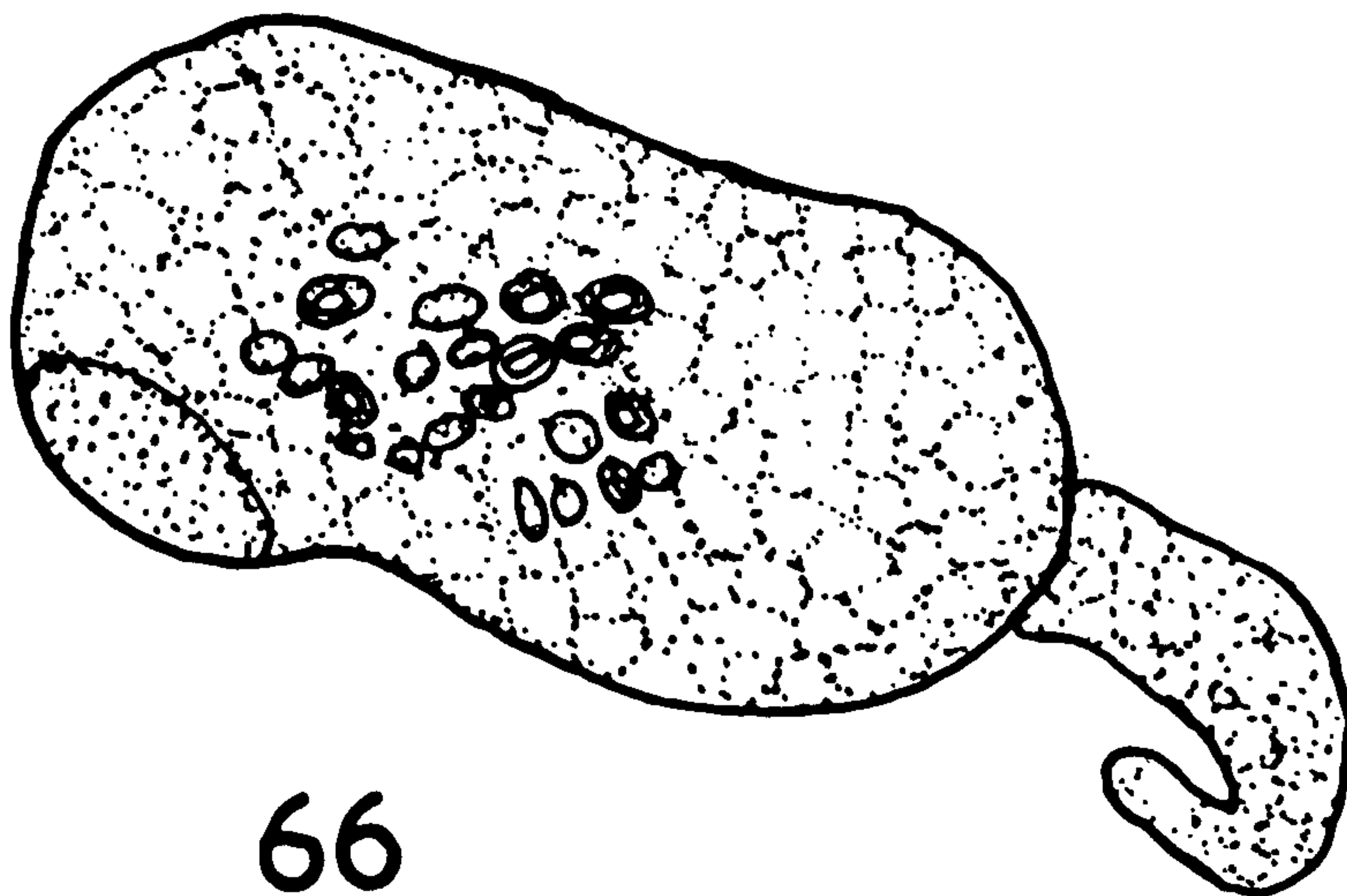
a fairly large mite and was thus easy to work with, this mite was used wherever possible in the experiments.

In the literature to date there is no record of Moniezia cysticercoïds developing in X. tereocranus either in nature or under experimental conditions. It was therefore necessary to see whether this mite could be infected with Moniezia experimentally. There was no intention of carrying out the experiment on a large scale due to lack of time and so only a small number of mites was used.

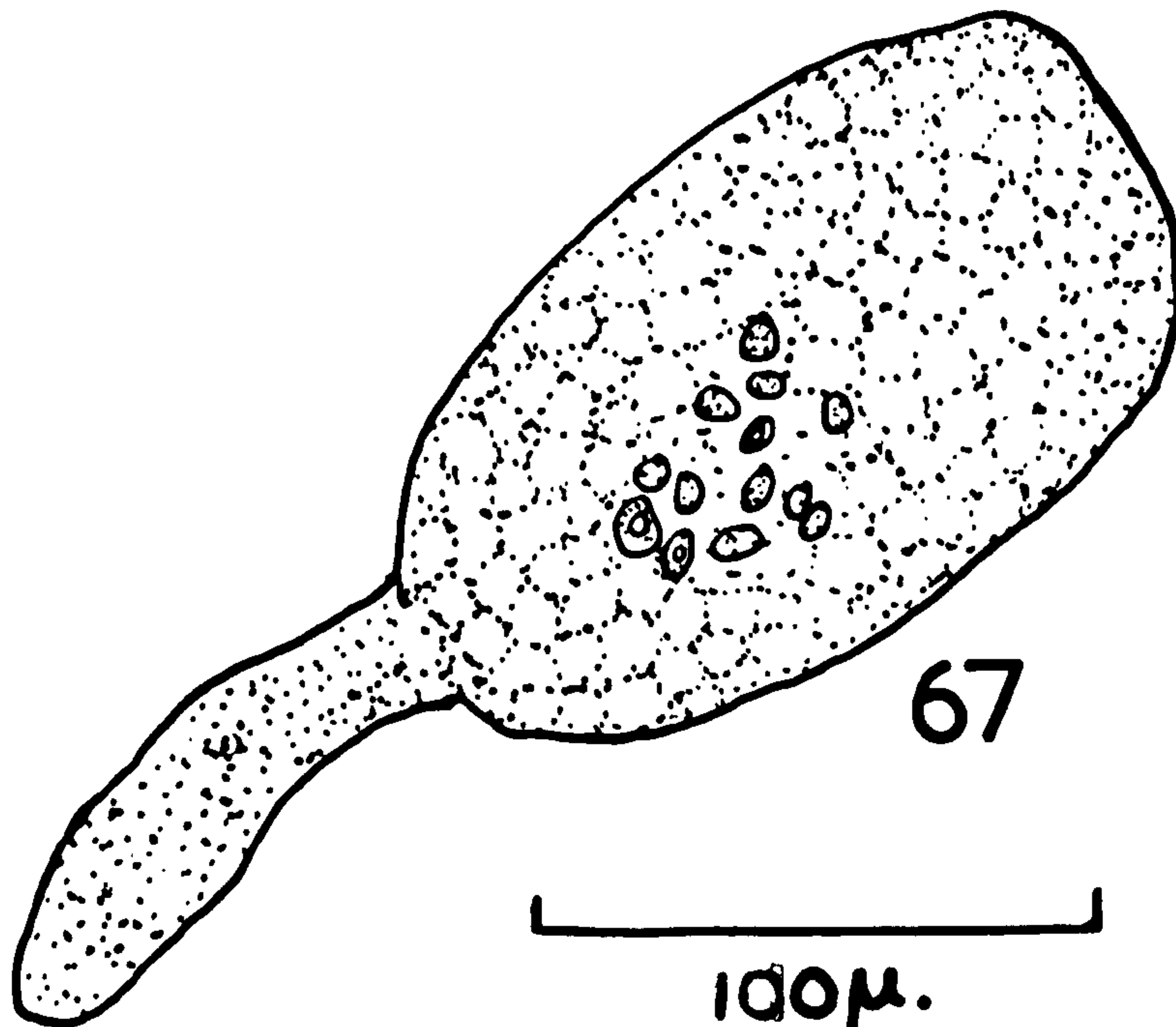
Sixty three specimens of X. tereocranus were placed in two sets of culture tubes, each tube containing six to ten mites. One set was kept as controls ( twenty nine mites) . The others were fed with eggs of Moniezia expansa either by adding gravid segments, or suspensions of eggs that had been kept in the refrigerator for about two weeks, into the culture tubes. The same was done with an assorted lot of mites ( 165 mites) of which ninety five were " Notaspids", about half of them being kept as controls. After a period of two weeks two mites from each set ( experimental and control) were examined each week for evidence of infection with Moniezia larvae. At



PLATE X



66



67

100μ.

Two cercomere stage larvae of Moniezia expansa from an experimentally infected Xenillus tegeocranus.  
s- sucker rudiment, c- calcareous bodies,  
ce- cercomere.

the eleventh week two mites from the lot of X.tegeocranus fed with eggs, showed cercomere stages, while none of the controls showed any. Of the two infected mites, one had three larvae with large cercomeres, calcareous bodies and a rudimentary sucker in one ( Plate X, Fig. 66-67). The other contained a single larva also at the same stage of development. This experiment was carried out on too small a scale and the number of infected mites was insufficient to carry out any other experiments that were desired. It showed, however, that Xenillus tegeocranus can be experimentally infected with larvae of Moniezia expansa.

It was next decided to see whether this species of mite (X.tegeocranus) and also other oribatid mites could be experimentally infected with N.helminthorum. A series of experiments, with controls, were performed where heavily infected segments of M. expansa were added to the culture tubes containing the mites. The results are given in the table below. It is seen from the table that two out of sixty five mites fed on spores of N. helminthorum, showed infection. One was a Ceratoppia bipilis and the other a Xenillus tegeocranus. The spores obtained from these mites were identical in shape and appearance with the spores of



N. helminthorum in Moniezias, but were of a smaller size. For instance the spores from C. bipilis measured in the fresh state were 2.7-3.1 by 1.25-1.5  $\mu$  ( Average 2.9 by 1.4  $\mu$  ), while the spores from X. tegeocranus, measured in sections, were 2.2-2.5  $\mu$  by 1.0- 1.25  $\mu$  ( Average 2.4 by 1.17  $\mu$  ).

Oribatid	No.	Duration	Results	
			Sections	Smears
<u>Platynothrus peltifer</u>	5	24 hours	2 -ve	3 -ve
"Notaspids"	3		1 -ve	2 -ve
<u>Xenillus tegeocranus</u>	2		-	2 -ve
"Notaspids"	4	48 "		4 -ve
<u>Hoploderma magna</u>	3		1 -ve	2 -ve
<u>X. tegeocranus</u>	20		5 6ve	15 -ve
<u>Ceratoppia bipilis</u>	8	6 "	3 -ve	5 -ve
"	1	7 "	-	1 +
"	2	8 "	1 -ve	1 -ve
<u>Scheloribates</u> sp.	1	8 "	-	1 -ve
<u>X. tegeocranus</u>	7	9 "	2 -ve	5 -ve
"	1	11 "	1 +	-
"	1	11 "	-	1 -ve
<u>C. bipilis</u>	2	12 "	-	2 -ve
<u>Belba</u> sp.	2		-	2 -ve
<u>Platynothrus peltifer</u>	1		-	1 -ve
<u>Euzetes globula</u>	2		-	2 -ve
<u>Controls</u>	-	<u>X. tegeocranus</u>	5 - ve	15 -ve
		<u>E. globula</u>	2 - ve	4 -ve
		<u>Platynothrus peltifer</u>	2 -ve	3 -ve

It was not possible in C. bipilis, to say from which part the spores came, because the spores were only seen after the mite was crushed. They were probably from the gut. In sections of the infected X. tegeocranus the spores were present in the epithelial cells of the mid-gut and caeca. The infection was so intense that many cells were hypertrophied and filled with spores. Some of these cells were nothing but spore-filled sacs a few of which had ruptured liberating the spores into the lumen of the gut. No developmental stages of the parasite were seen in these sections, nor was there any evidence of the infection having spread to other structures in the surrounding body cavity.

Figs. 68 and 69 (Plate XI ), show sporonts and spores seen in a dry-fixed smear of the infected C. bipilis. One spore is shown with an extruded filament. ( Fig 68). The sporonts show the characteristic ' pre-metachromatic granule' which was seen in sporonts of N. helminthorum in Moniezias. ( Fig. 69 b ). A photomicrograph of the infected X. tegeocranus (Fig. K ) shows a heavily infected epithelial cell of the mid-gut that has ruptured liberating the spores into the lumen.



Explanation of Plate XI

Spores and sporonts from smear of experimentally infected Ceratoppia bipilis ( Giemsa ).

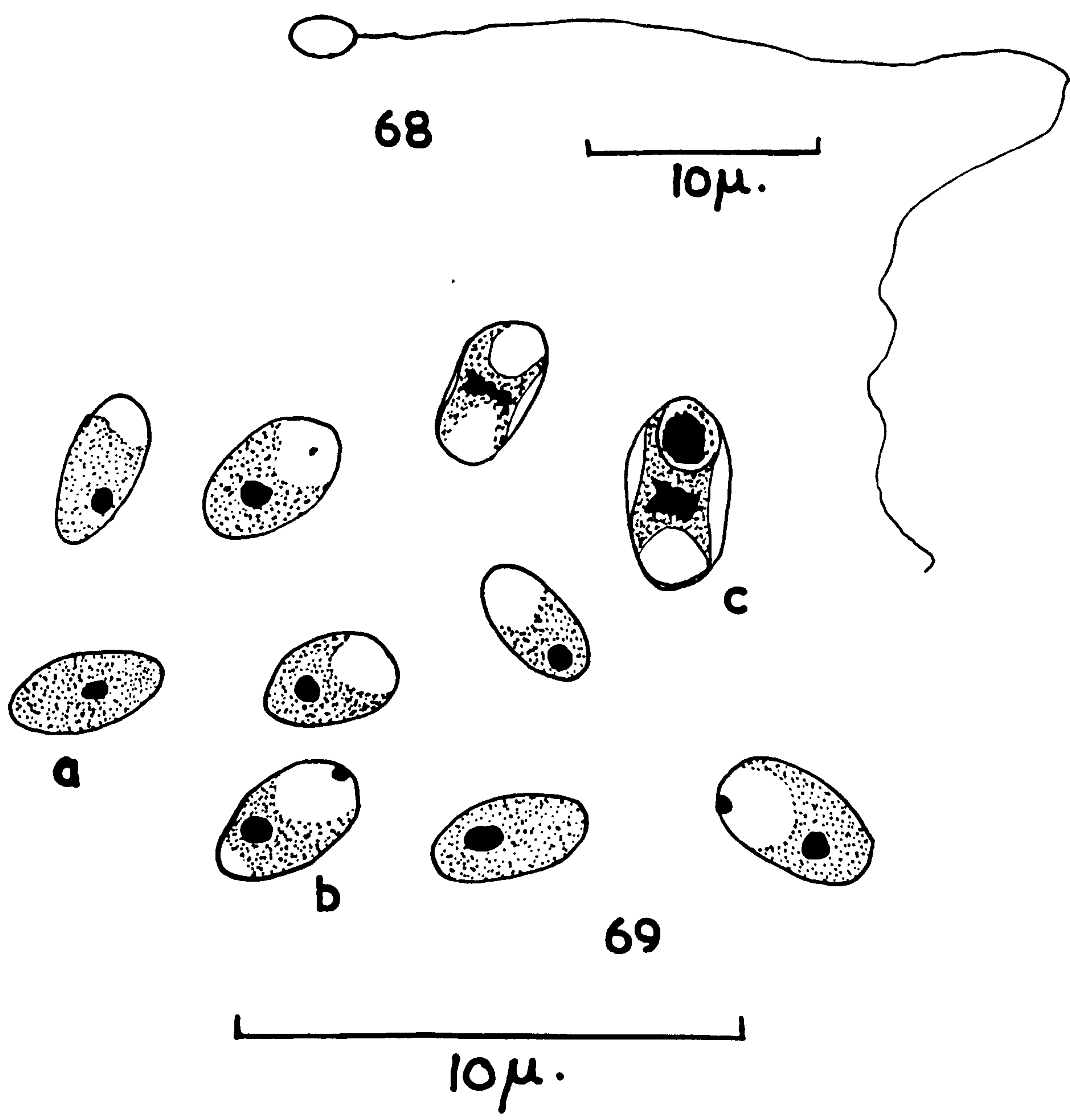
68. Spore with extruded filament.

69. Sporont stages and spores.

a. Early sporont.

b. Sporont with 'premetachromatic granule' .

c. Mature spore showing nucleus and  
metachromatic body.





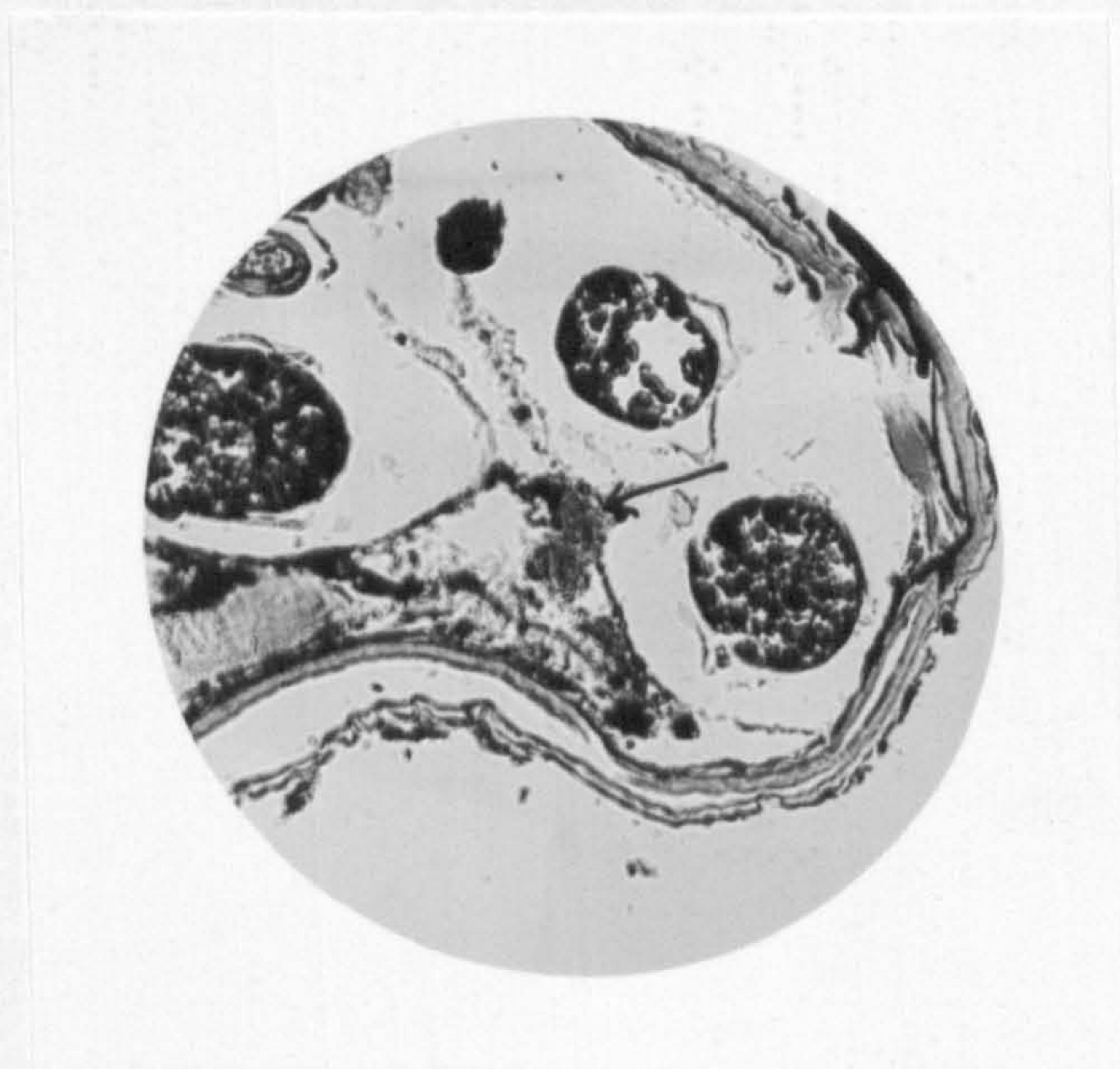


Fig. K.

Section of Xenillus tegeocranus infected with Nosema helminthorum, showing spores in hypertrophied epithelial cell of mid-gut. Some spores are seen liberated in the lumen by rupture of cell. (Giemsa-Colophonium)  
( X 225 )



## Discussion

From the absence of any records of microsporidian infections in oribatid mites in the literature, and the failure to find any natural infection with this group of protozoa, it may be concluded that these oribatid mites probably do not possess and hence do not transmit their own microsporidian to the larval stages of Moniezia. However, the results of the last series of experiments are interesting since they indicate that N. helminthorum is capable of infecting mid-gut cells of these oribatid mites. This brings us to the question of the host range of N. helminthorum which, as will be seen later, infects a few cestodes belonging to different families. As far as oribatid mites are concerned, their susceptibility to this microsporidian may indicate that the microsporidian may have at one time been a parasite of these mites, and that the Moniezias have acquired the infection from them due to their close connection with these mites. So far the only anoplocephalid cestodes from which N. helminthorum has been reported are the Moniezias, N. expansa and M. benedeni, but it is quite possible that they may eventually be found in many other tapeworms of this family.



## Chapter VI

### Experimental infections of various Cestodes

#### 1. Experiments with Hymenolepis nana of Mice and Rats.

In considering the mode of transmission of Nosema helminthorum from one Moniezia to another the following possibilities were examined.

(a) Oribatid mites might play some role in the transmission by transferring the microsporidian to the larval stages of the cestode, or by carrying the spores within their gut lumen, thus protecting the spores from the gastric juices of sheep.

(b) A sheep already harbouring Moniezia in its intestine may accidentally swallow spores of the microsporidian found contaminating grass in the grazing ground.

The first of these possibilities has already been investigated and it has been seen that the only part that the mites might play is to mechanically transfer the spores into the small intestine of a sheep in which

the adult worm is already present. It was therefore decided to see if spores of N. helminthorum could be made to infect Hymenolepis nana of mice and rats by feeding them to these animals which were already known to harbour the worms. By this method the second of the above possibilities could be tested in the laboratory. The following series of experiments were therefore performed.

Experiment 1. Infection of H. nana of mice with  
N. helminthorum.

Five mice examined previously and found to be negative for H. nana, were fed eggs from gravid segments of H. nana on 10.6.54. On 23.6.54, all these mice were found to be passing eggs of H. nana in their faeces. These mice were then separated into two jars. The first jar containing three mice was kept as a control. The two mice in the other jar were fed spores of N. helminthorum, by feeding them forcibly on heavily infected segments of M. expansa. This was repeated on 24.6.54 and 25.6.54. On 28.6.54, five days after the first feed, one of the mice in the experimental jar was sacrificed, and twenty worms recovered from it. Fifteen of these worms were examined carefully by crushing



the segments in saline and studying under the high power of the microscope. Five of these worms showed ovoidal spore-like structures, which bore a strong resemblance to immature spores of N. helminthorum, but were of a smaller size. They were pointed at one end, hyaline and only slightly refractile. The vacuole at the posterior broad end was quite prominent and occupied a greater volume in proportion to the size of the spore. It was obvious that the spores were those of N. helminthorum of a smaller size than usual. But in order to prove this definitely it was necessary first to examine the worms from the control mice, and secondly to repeat the experiment several times more. The smears in which the immature spores were detected were dry-fixed and stained in Giemsa stain. Many sporonts and immature spores were seen (Plate XIII, Fig. 72-74). The sporonts showed the pre-metachromatic granule so characteristic of N. helminthorum.

The five remaining worms were fixed in Carnoy's Fluid and sectioned at 4  $\mu$ . thickness. Three of them showed evidence of infection, as spores and earlier developmental stages were seen scattered in various places in the parenchyma of the worms. One worm showed in the scolex and neck regions, second phase

fusiform schizonts some with double nuclei , as was seen in W 125 and M 135. (Plate XIII, Fig. 84).

On 2.7.54 one of the control mice was killed and thirty three worms recovered from it. Twenty of these worms examined by crushing were negative. Three were found negative after sectioning, while the remaining ten were fixed in Formo-saline and later stained by Newton's method. This was found to be a useful and delicate method for detecting spores of the microsporidian even when only a single spore was present in the entire worm. No sign of infection was present in these worms. Thus it was clear that none of the control worms had any infection of this sort.

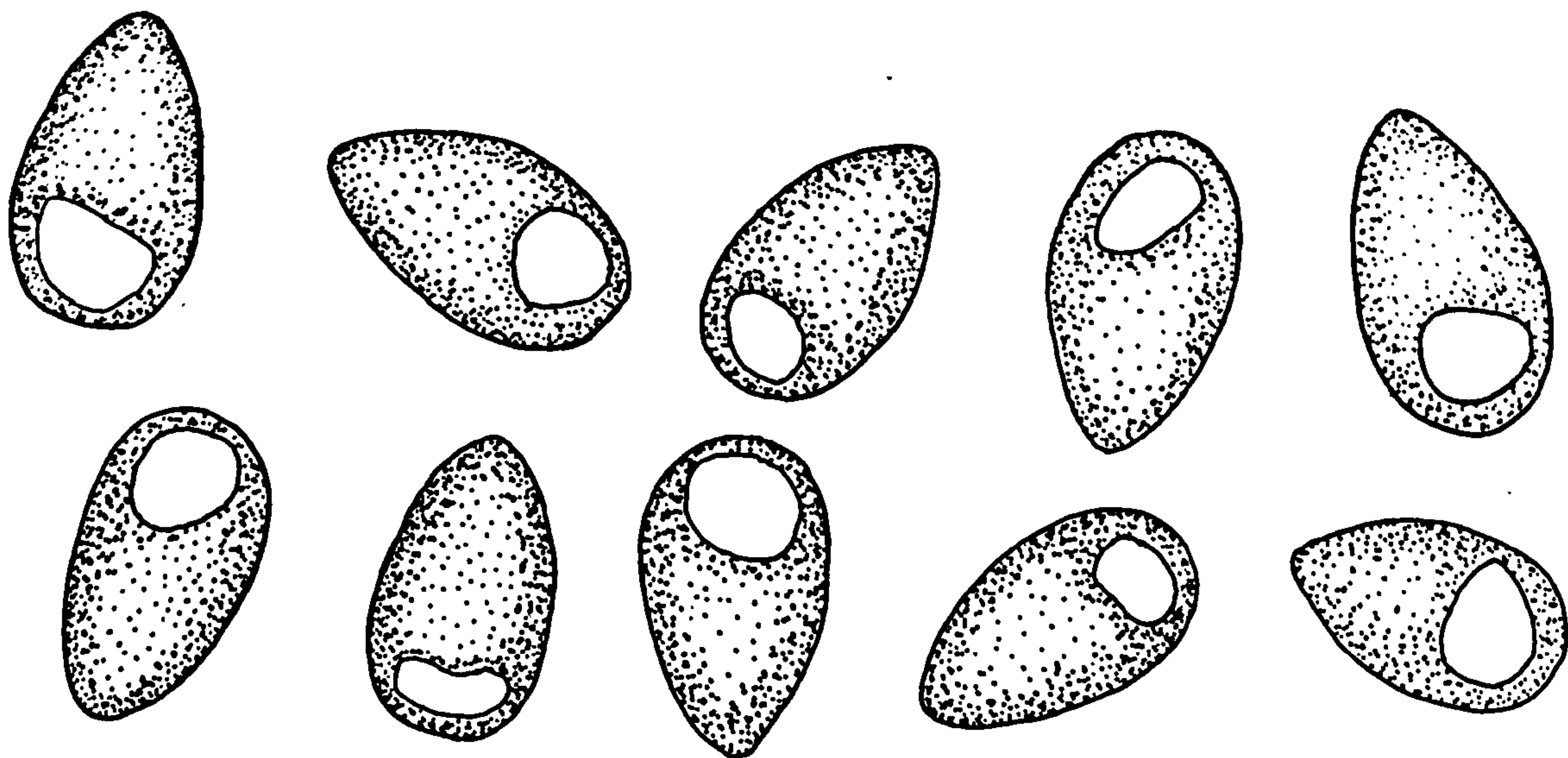
On 5.7.54, twelve days after the first feed, the second experimental mouse was killed. The last three inches of its small intestine were teeming with numerous small-sized specimens of H. nana . Eight out of sixty-four of these that were examined by crushing in saline showed mature spores of the microsporidian . These were identical in appearance with those of H. helminthorum in Koniezias but of a smaller size ( Plate XII, Fig. 70 ). They measured in the fresh state 5.0- 6.2  $\mu$ . by 2.9-3.5  $\mu$  by ( Average 5.7 by 3.2  $\mu$ . ). Polar filament extrusion



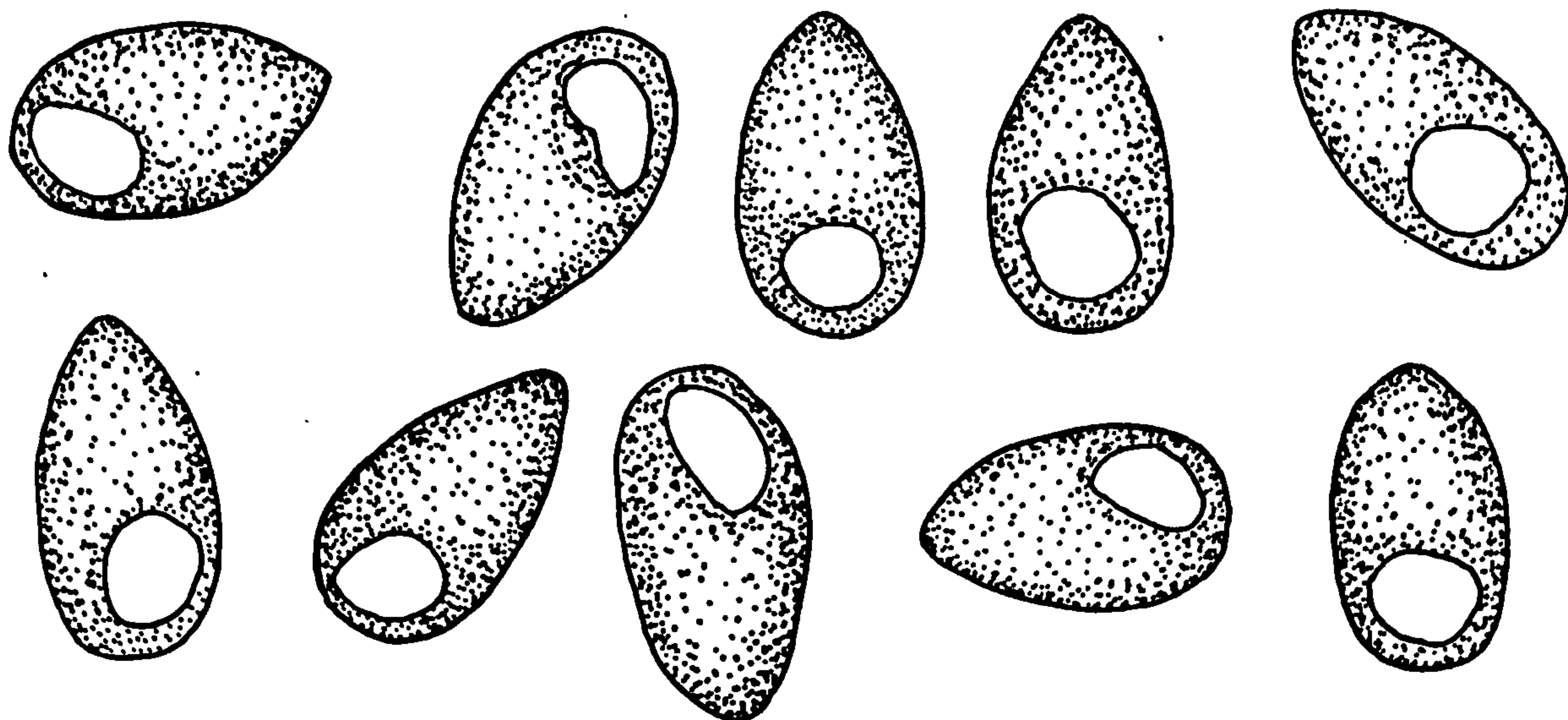
Explanation of Plate XII

70. Fresh spores from a Hymenolepis nana  
infected with spores from Moniezia expansa.

71. Fresh spores from a Hymenolepis nana  
infected with spores from an experimentally  
infected H. nana.



70



71



was quite easily accomplished by the mechanical pressure method of Kudo, and the filaments measured 70- 105  $\mu$ . ( Average 80  $\mu$  ), in length.

Thirty-three of the fifty-nine worms stained by Newton's method showed spores. Some of these appeared to have only a few scattered spores, and it is quite possible that such a high percentage of positive worms would not have been obtained if this method had not been adopted. Fig. L, shows one of these worms stained by Newton's method.

A portion of the intestine of the second experimental mouse containing worms 'in situ' was sectioned. Although a large number of sections was examined there was no trace of infection of the intestinal mucosa with this microsporidian, inspite of the fact that several infected scolices were seen embedded in the mucosa.

The two remaining control mice were killed on 5.7.54 and 30 worms were recovered from one and thirty - five from the other. All were negative after examining by the Newton method.

Experiment 2. Infection of M. nana with spores from M. expansa.

It was decided to repeat the above

experiment keeping the infection for a longer period. It was soon found that many of the mice in the stock were already infected with H. nana, and so it was not necessary to infect them. Faecal examination of several mice in the stock was made and those showing H. nana eggs were used for this and subsequent experiments. In this experiment one mouse was kept as control, and two were fed on spores in infected segments, and suspensions of spores sprinkled on the food, on 7.7.54 and 8.7.54. The first of these mice was killed on 17.7.54, ten days after the first feed and over thirty worms were recovered from its intestine. At the same time large numbers of oxyurids were recovered from the caecum. These proved to be specimens of Aspiculuris tetraptera. Two out of the thirty tapeworms were crushed in saline and both showed spores as well as sporonts. Nine out of eleven examined by Newton's method showed infection, while all ten worms that were sectioned showed spores and other developmental stages.

None of over fifty oxyurids from the caecum of this mouse showed any signs of spores. They were examined in saline and many of them were crushed and examined in great detail. These worms had obviously



been exposed to infection with the spores of N. helminthorum but failed to get infected.

The second experimental mouse was sacrificed on 27.7.54, nineteen days after feeding it with spores. Three out of three worms crushed and examined in saline showed large numbers of mature spores. Five out of eight stained by Newton's method, and three out of four that were sectioned, showed infection.

All the worms from the control mouse (which was sacrificed on 29.7.54) were negative, four being examined by crushing in saline and ten by Newton's method. Several specimens of Aspiculuris tetraptera from the caecum of this mouse were examined but showed no infection of any sort.

Experiment 3. Infection of H. nana with spores from an experimentally infected H. nana.

In this experiment an attempt was made to see whether spores developing in an experimentally infected H. nana are infective to a fresh set of worms. Accordingly three more H. nana-infected mice were used. One of these was used as a control. The other two were fed on 26.7.54 with infected segments of H. nana obtained

from experiment 2. The results were disappointing. One of the mice sacrificed on 9.8.54, fourteen days after feeding, contained no worms. The other had only two worms both of which proved to be negative.

The control animal was killed on 11.8.54 and none of the eight worms recovered from it showed any infection, three being examined by crushing and five by Newton's method.

As the failure of this experiment might well have been due to the small number of worms recovered, it was decided to repeat it later ( Experiments 6 & 7 ).

Experiment 4. Infection of H. nana with refrigerated spores of N. helminthorum from N. expansa.

Only two mice were used in this experiment, one being separated as a control. The other was fed on 10.8.54 with infected segments of N. expansa that had been left for over one month in the refrigerator in saline and in distilled water. The purpose of this experiment was to see whether spores thus stored are still infective. This would then be a helpful method of having a ready stock of spores for future infection



experiments when spores were suddenly required, as in the human experiments. The experimental mouse was killed on 23.8.54, thirteen days after feeding. Thirteen worms were recovered from this mouse and out of five examined by crushing, one was positive, while one out of the remaining eight stained by Newton's method was positive. The control animal was killed on 24.8.54. and six worms were recovered. Three of these were examined by crushing and three by Newton's method. None of them showed any infection.

From the results of this experiment it was clear that spores stored in this way in water or saline and left in the refrigerator for long periods could be used with advantage whenever the need arose.

Experiment 5. Repetition of experiments 1 and 2.

In this and some subsequent experiments no controls were used because it had become evident that the infection in the experimental mice worms was definitely due to the spores administered to these mice. The main purpose of this experiment was to get sufficient quantities of spores from H. nana for experiments 6 and 7, - the repetitions of experiment three.



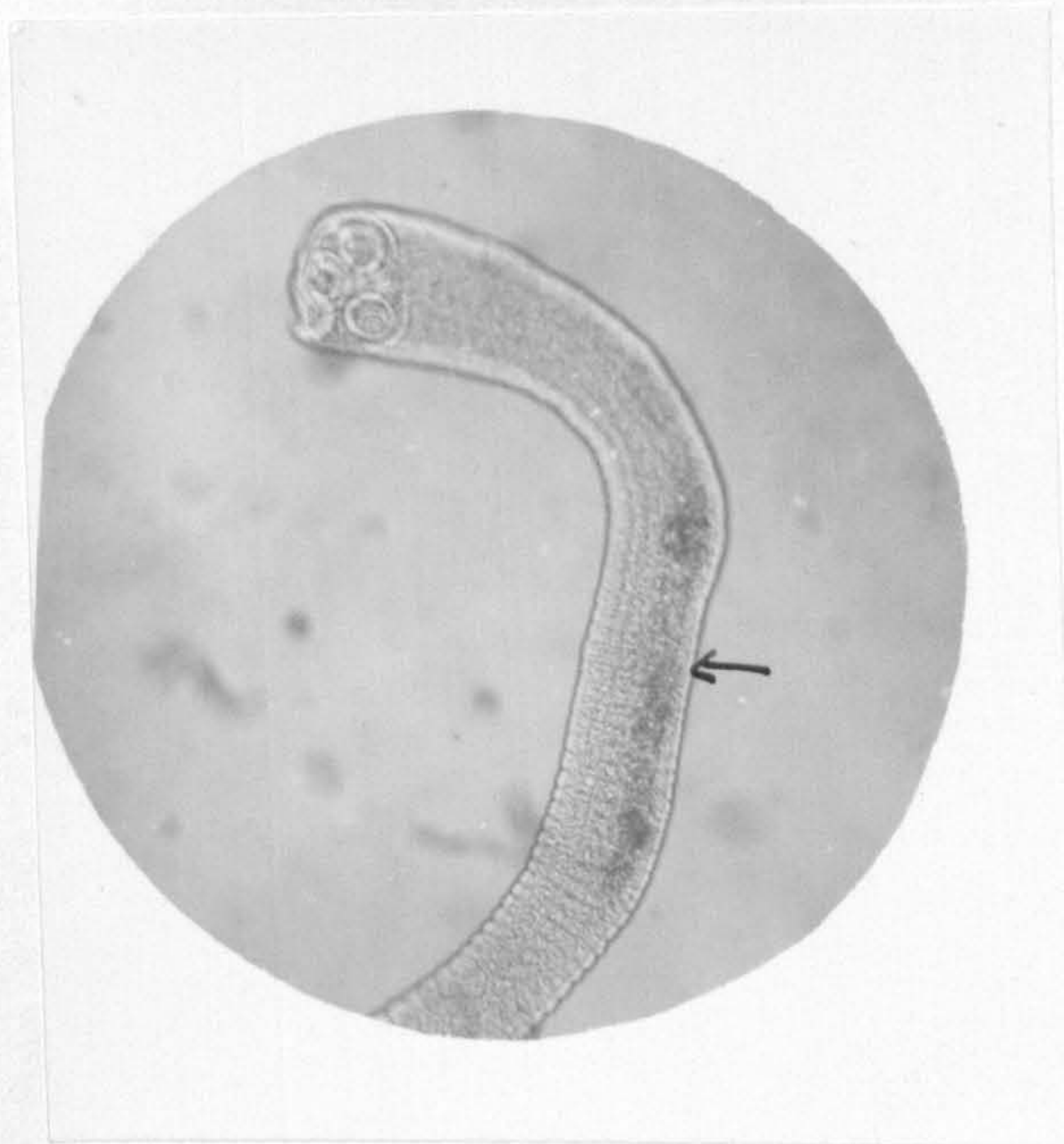


Fig. M L.  
Hymenolepis nana experimentally infected with Nosema helminthorum, stained by Newton's method to show up the spores. ( X 80 )



Fig. M.  
 Section of a heavily infected H. nana ( H.S. ) showing spores filling all tissues, but the eggs unattacked. ( Giemsa-Colophonium X 125 )



Three mice were fed with spores from a heavily infected M. expansa on 11.8.54, by forcibly feeding the segments, and later adding suspensions of the spores on to the food pellets of the mice. One of these mice was killed on 20.8.54, nine days after feeding the spores. Nine worms were recovered from it. Four out of five of these were positive by crushing, and one out of two examined by Newton's method. Two worms were fixed in Carnoy's Fluid and sectioned. The sections showed all stages from early schizonts to spores ( Plate XIII, Fig. 76-83 ). The spores from the four positive crushed worms were used in Experiment 6.

The second mouse was sacrificed on 6.9.54, twenty six days after feeding the spores. All twenty-four worms that were recovered from it were packed with spores of the microsporidian. One of these worms was fixed in Carnoy's Fluid for sectioning. The sections showed heavy infection with spores which packed all tissues in the cortex and the medulla, but the ovaries and testes were unattacked ( Fig. N ).

Twenty five oxyurids recovered from the caecum of this mouse were examined in great detail but showed no spores. They too were specimens of Aspicularia tetraoptera.

The third mouse was killed on 7.9.54, twenty-seven days after feeding spores and once again all twenty-five worms that were recovered were packed with spores. The infection could therefore be detected with the low power of the microscope. About half an inch of the intestine of this mouse was sectioned with the worms 'in situ'. In no portion of the intestine was there any trace of spores. Two individual worms were also sectioned and they both showed heavy infection with spores, the genital glands being once again unattacked.

The stools of the second and third mice were examined at intervals after the second week of infection, and they both showed spores in large numbers. These were obviously spores liberated from the detached gravid segments. Most of the worms from these last two mice were fed to the mice used in Experiment 7.

Experiment 6 .Infection of H. nana with spores from experimentally infected H. nana.

A single H. nana-infected mouse was fed segments of infected worms from mouse 1 of Experiment 5, on 20.8.54. It was killed on 1.9.54, twelve days after feeding. None of four worms examined after crushing in saline showed any signs of infection. One



worm was sectioned after fixing in Carney's Fluid, and it showed a few spores. No controls were used in this.

Experiment 7. Repetition of Experiment 6.

Since the results of experiments 3 and 6 were not very convincing it was decided to carry out one more experiment on these lines. Three mice were used one of which was kept as a control. The other two were fed the spore-filled segments of H. nana from the last two mice in experiment 5. The first of these mice was killed on 20.9.54, fourteen days after infection, and the second was killed thirty-two after. Only four worms were recovered from the first, and one of them showed spores in crushed preparations. Six worms<sup>were</sup> recovered from the second mouse and all were infected.

The control mouse was killed on 12.10.54 and none of the five worms from it showed infection.

A dry-fixed smear preparation made out of one of the infected worms showed several sporonts and immature spores in addition to mature spores. Some of the spores left to dry for one hour and subsequently moistened with distilled water showed extrusion of filaments. A few of the filaments showed a globular

body at the tip ( Plate XIII, Fig. 75 ). In some spores in which this body was absent at the tip of the filament it was apparently left behind at the anterior end of the spore when the filament was extruded.

From the results of this experiment it became quite clear that the spores of N. helminthorum developing in H. nana are quite normal and infective to other H. nana. The spores appearing in the latter were similar in size and appearance to those originally fed (Plate XII, Fig. 71) and measured 5.7-6.0  $\mu$  by 3.0-3.4  $\mu$  ( Average 5.8 by 3.2  $\mu$  ). These measurements show that the spores derived from an experimentally infected H. nana maintain their size even when they infect a fresh set of H. nana.

Experiment 8. Attempts at observing the 'trans-cuticular' stage.

The fact that in previous experiments spores of N. helminthorum fed to H. nana-infected mice, could produce an infection in these worms suggested that the sporoplasm must work its way into the tissues of these worms through the cuticle. The mode of emergence of the sporoplasm has been shown earlier to bring the sporoplasm in contact with the surface of the host tissue. There is no doubt that the filament cannot penetrate the thick



cuticle of tapeworms. It is obvious therefore that the sporoplasm must work its way in by secreting some histolytic substance. That microsporidia can penetrate thick helminth cuticle in this manner has been shown by Thorne (1940). He found that a microsporidian, Duboscqia penetrans, parasitic in a free living nematode Pratylenchus pratensis, attacks the cuticle of the helminth in its external phase and the body cavity in its internal phase. He said that in internal parasitisation the parasite finds its way into the body cavity by penetrating the cuticle. He was unable to observe the filaments of this organism and from his figures it is not certain whether it is a microsporidian at all, but it shows that such a penetration of the cuticle of helminths by protozoa is possible.

In this experiment therefore an attempt was made to see if this cuticle- penetrating stage could be seen. Four mice were used, all of them being previously examined for the presence of H. nana ova. No controls were used. The mice were all fed infected segments of M. expansa at 4.30 p.m. on 8.9.54. They were killed at the following times on the next day.

Mouse 1.	12.30 p.m.	-	20 hours.
" 2.	4.30 p.m.	-	24 "

Mouse 3. 5.30 p.m. - 25 hours.  
" 4. 12.30 p.m. on 11.9.54 - 68 hours.

One worm was recovered from mouse 1. This was fixed in Bouin's Fluid for sectioning. Mouse 2 had no worms. Mouse 3. had several worms, one of which was fixed in Bouin's Fluid for sectioning. The rest were fixed 'in situ' (for sectioning) with the intestines. Two out of the three worms recovered from mouse 4 examined by crushing showed several sporont stages of the microsporidian. The remaining worms from mouse 4 were fixed in situ with a piece of the intestine, for sectioning.

Results The results were rather disappointing since none of the sections of worms from mice 1 and 3, showed any stages of the parasite at all either in the cuticular region or elsewhere. In the sections of worms in the intestines of mouse 4 only a few scattered solitary sporont stages were detected. These results show that within sixty-eight hours the development in H. nana can proceed to the sporont stage. Furthermore in this instance no marked schizogonic multiplication had taken place and the sporonts were formed very rapidly after the initial infection. This again showed, as was seen in some Moniezias, that overcrowding with developmental stages of the parasite is



is not the important factor in bringing about spore formation, but that some other unknown factor is involved. It appeared in fact from this case that the original sporoplasms had migrated into the deeper tissues of the tapeworm, some even to the medulla, and had directly transformed themselves into sporonts. Alternatively they had undergone only a very short period of schizogony before giving rise to sporonts. In a few sections a more or less rounded deeply-staining body was seen just under the cuticle between the subcuticular cells. This appeared to be a very early stage of the parasite, but as nuclear details were not recognisable this was regarded as inconclusive.

Experiments 9 and 10. Further attempts at demonstrating the cuticle-penetrating stage.

Owing to the failure of the above experiment the next two experiments were conducted in the following manner.

Experiment 9 -- One mouse was fed continuously with the spores of N. helminthorum by adding a concentrated emulsion containing the spores to the drinking water. By this method it was hoped that whenever the mouse was killed it would be possible to see at least a few early

penetrating forms. This mouse was killed on 14.9.54 three days after the spores were introduced into the drinking water. Unfortunately no worms were recovered from this mouse.

Experiment 10. The same procedure was adopted in this experiment. Two mice were first fed a very large quantity of spores by forcibly feeding over ten heavily infected segments. This was followed by adding a suspension of spores into the drinking water. This experiment was begun at 3.30 p.m. on 10.11.54. From Experiment 8 (mouse 4) it became evident that the sporoplasm must penetrate the cuticle at a much earlier stage, and so the first mouse was sacrificed after  $2\frac{1}{2}$  hours. There were over a hundred worms in this mouse which were higher up in the intestine than they normally are. A few of these worms were fixed in Carnoy's Fluid for sectioning. Several portions of the intestine with the worms 'in situ' were also fixed for sectioning. Before this the contents of various parts of the alimentary canal of the mouse were examined in detail under the microscope to see the condition of the spores in these various sections of the gut. The stomach showed a large number of spores free from the segments. About 50 % of these were empty, but there were no traces of any



filaments. In the upper parts of the small intestine the proportion of empty spores was decidedly greater, while in the last portions of the small intestine where the worms were present there were few spores which had not extruded their filaments. Amongst some of these worms the spores with their extruded filaments were seen in an entangled mass. A few of these worms with some of the intestinal contents were kept under a sealed coverslip for a few hours, and it was noticed that the filaments soon disappeared. The greatest number of spores was found in the caecum and practically all were empty. No filaments were seen in this part of the gut. From these observations it appears that extrusion of the filaments takes place in all regions of the gut of the mouse, and that they are soon dissolved by the digestive juices. Zwölfer (1926) has shown, for Plistophora blochmanni, that trypsin dissolves the filaments in twenty four hours.

It would appear from this that the filament, in contrast to the spore wall, is definitely not made up of a resistant material as Minchin (1912) believed. Therefore the function of the filament must be only temporary as has been suggested in the section on the emergence of the sporoplasm.

The second mouse was killed after five hours and the gut contents were similarly examined. It was found that the same conditions obtained here too. Only three large worms were recovered from this mouse and they were fixed in Carnoy's Fluid for sectioning.

Results. - In none of the worms sectioned singly or 'in situ' in the gut obtained from the first mouse, were any stages of the parasite seen, although several serial sections were carefully examined. The same was true of the three worms sectioned from the second mouse.

#### Discussion.

Within two hours a large number of spores that have extruded their filaments and are to be found in the region of the intestine where the worms are present. This shows that quite a few of the sporoplasms must have come in contact with the cuticular surface of the worms. It is surprising therefore that no cuticle-penetrating stages were seen. However, it must be remembered that the chances of missing the relevant portions of the worms are great, and also that the so called planont stage has not clearly been demonstrated in any of the microsporidia, although many workers have claimed that they have seen this stage.



Experiment 11.

This experiment was performed during attempts at elucidating the nature of the fusiform cells in M 125 and M 135. It was thought at one stage that these cells may represent a sensitivity reaction on the part of the host to a second infection. Six H. nana-infected mice were given large numbers of infected segments of Moniezia on 27.10.54. One lot of three mice was then fed a second dose of spores after thirteen days (on 9.11.54). The other three mice that had only one dose, were kept as controls. Two of the re-infected mice were killed on 19.11.54 and the third on 25. 11. 54, ten and sixteen days respectively after the second dose. Two worms were recovered from the first mouse and one each from the other two. All the control mice were killed the following day and four worms recovered from them were sectioned. Sections of all the re-infected worms showed the cortical and medullary regions packed with spores and in addition numerous sporont and pre-sporont stages. There were no signs of a 'sensitivity' reaction to the second infection. The control worm sections too showed the tissues packed with spores but no sporont or earlier developmental stages were present. An important finding

in this experiment which confirmed the findings in Experiment 5 (mice 2 and 3 ) was that when the infection was so heavy that it involved the whole cortical and medullary parenchyma, the membranous partitions of the genital glands were not broken through(Fig. M ). This means that the chances of trans-ovarian infection are remote as is also the case in infected *Moniezia*s. This will be discussed fully later.

#### Experiment 12.

In this final experiment with *H. nana* of mice an attempt was made to see the effect on the infectivity of the spores, of centrifuging and refrigeration. This was considered a suitable method for preserving the spores for later experiments. Some heavily infected segments of *Moniezia* were ground in a mortar and filtered through a fine sieve. The filtrate was then centrifuged at 2500 revolutions/minute for three minutes. The deposit now contained a very heavy collection of spores which were kept in the refrigerator for one week. This was then fed to one mouse infected with *H. nana* , another mouse being kept as a control. On 25.11.54, six days after infection both mice were killed and the worms recovered from



them. The experimental mouse had five worms, four of which were infected, showing spores and sporonts in crushings of the worms. None of the eight worms from the control mouse were infected.

The result of this experiment showed that centrifuging the spores does not affect their infectivity, and this method was used for storing some of the spores that were later used in the human tapeworm experiments.

Experiment 13. Infection of H. nana of the rat.

One rat infected with H. nana was fed infected segments of Moniezia on 26.11.54 and killed on 7.12.54, eleven days after. All of the six worms recovered from this rat showed spores and other developmental stages, chiefly sporonts, as with H. nana of mice. The spores had the same appearances and the measurements were 5.7 -6.3  $\mu$  by 2.9- 3.5  $\mu$  (Average 5.9 by 3.2  $\mu$  ).

A single control rat that was used in this experiment was also killed on the same day, but showed no infection in any of the three worms recovered from it.

### Explanation of Plate XIII

Stages of development from experimentally  
infected H. nana of mice.

72. Uninuclear schizonts from smear preparation.

73. Binuclear " " " "

74. Sporont stages from smear preparation.

75. Spore with extruded filament showing sporoplasm  
at the tip.

76. Early primary phase schizonts from sections.

77) Late " " " "  
78)

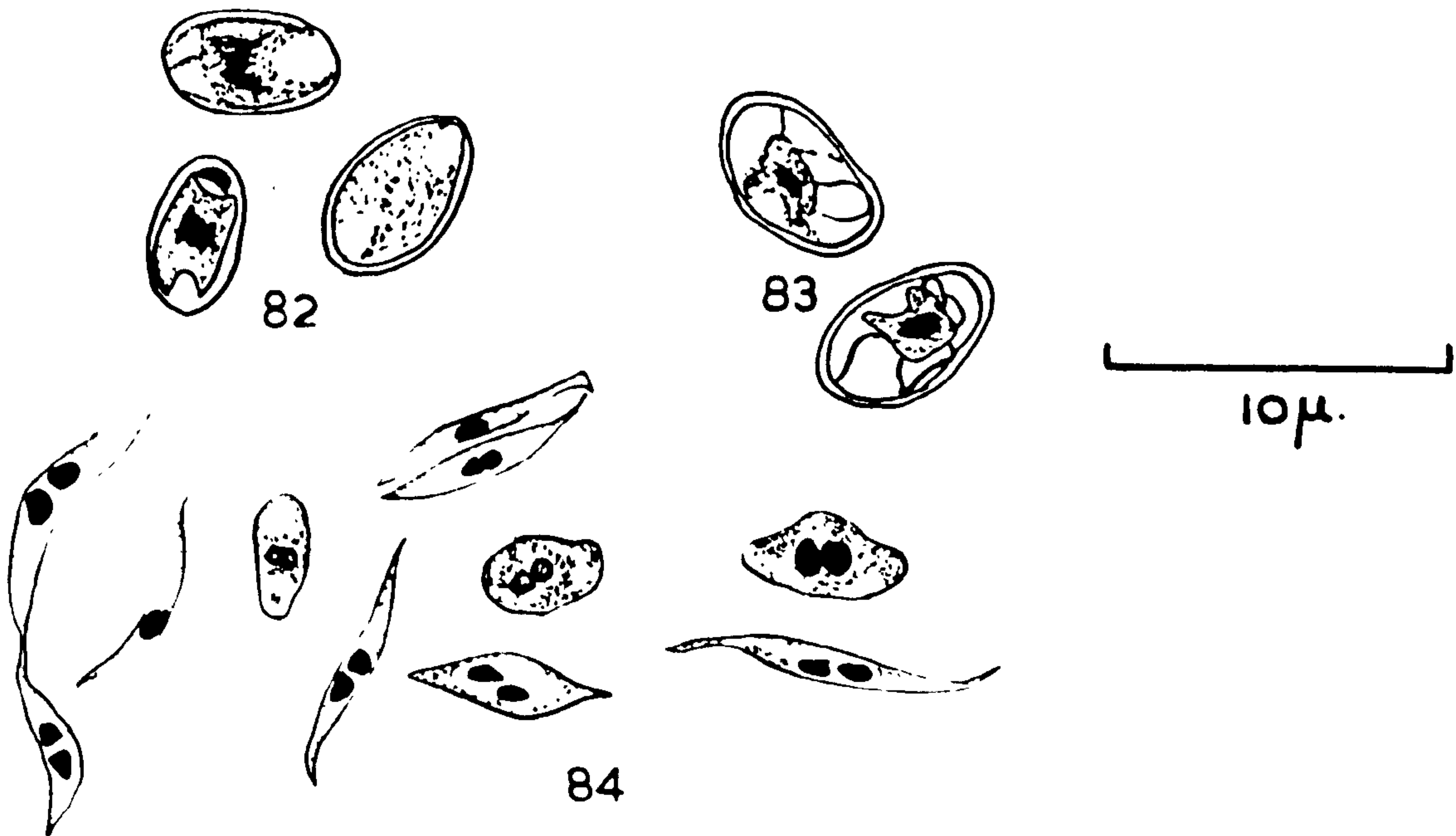
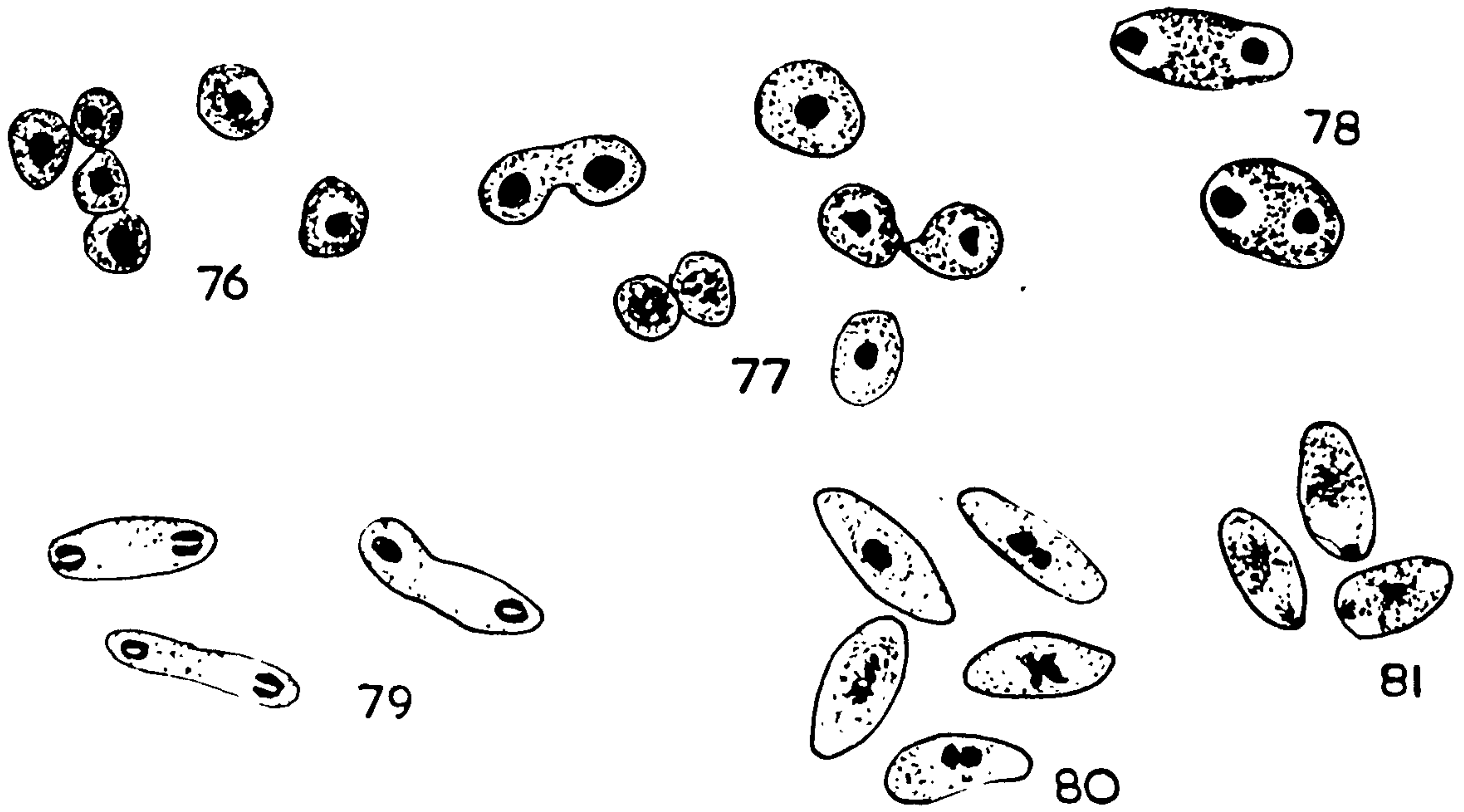
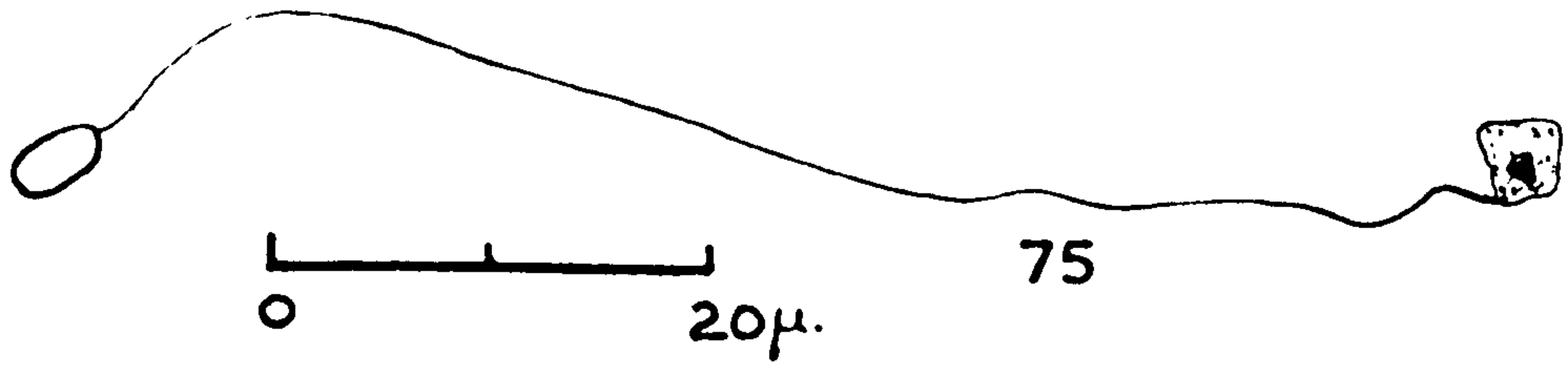
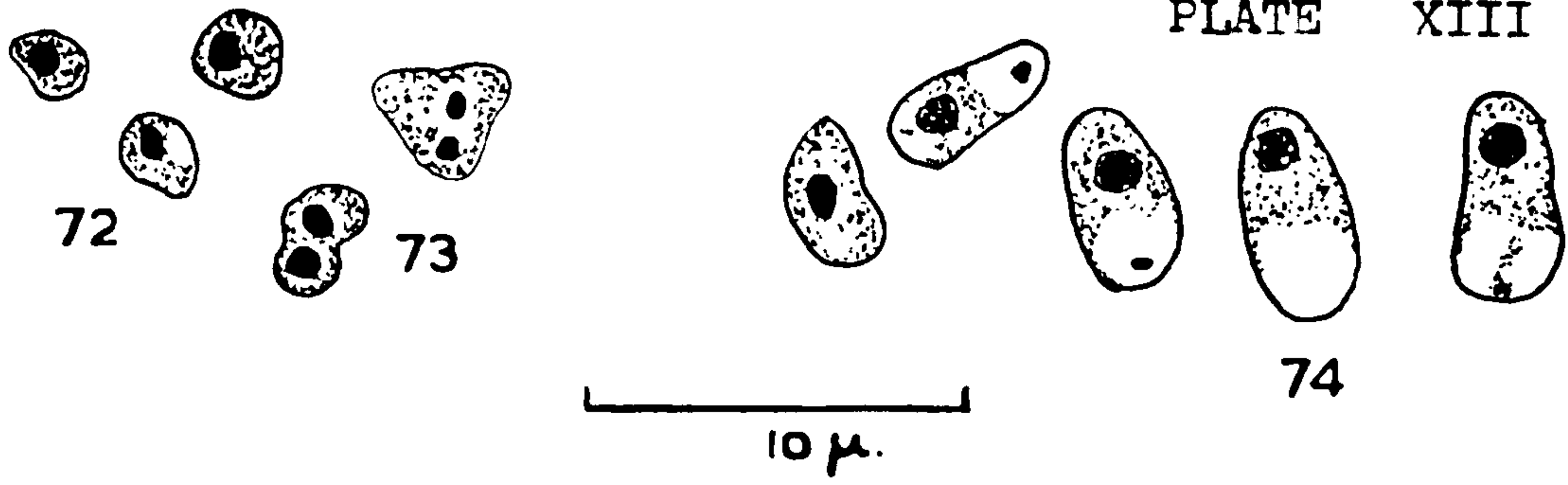
79. Early secondary phase schizonts ".

80-81. Sporont stages .

82 & 83. Spores stained in Giemsa ( Sections)

84. Various fusiform schizonts and sporonts  
from the scolex region of an infected H. nana.





Resumé of the development of Nosema helminthorum in Hymenolepis nana of mice and rats .

It is seen from the above experiments experiments that Nosema helminthorum readily infects Hymenolepis nana of mice and rats and develops quite normally in them giving rise to spores that are infective to fresh worms within three to five days. The stages of development resemble those that occur in Moniezias, except for the fact that they are on the whole smaller than the corresponding stages in Moniezia. It will therefore suffice to give only a brief account of the different stages that were met with in H. nana.

The earliest stage seen in sections is a spherical body of about  $1.3 \mu$  diameter with deep-staining cytoplasm and compact nucleus. ( Plate XIII, Fig. 76). It appears in the mice-worms within 24-48 hours of feeding the spores to mice. These are followed by schizonts with less deeply staining cytoplasm and in some of them the nucleus is surrounded by a clear zone as was seen in the corresponding stages in Moniezia (Plate XIII, Fig. 77,78). Stages in binary fission (Fig. 77) and stages corresponding to binucleate schizonts that probably divide by cytoplasmic cleavage are also observed ( Fig.73,78)



In one infected worm elongate schizonts with two nuclei at opposite ends were seen (Fig. 79). In some of them each of the two nuclei had a double appearance. These correspond, no doubt, to the earlier stages of second phase schizogony. Fusiform cells closely resembling the cells seen in M 125 and M 135 were seen in a few worms, (Experiment 1). In them the double nuclei which remained in close contact after dividing, were clearly recognised, together with all stages from autogamous schizonts to sporonts and spores (Fig. 84). The fusiform cells were only seen in worms from Experiment 1, but in others, stages represented in Fig. 80 and 81 were the more common precursors of spores. These sporonts in some cases had double nuclei, while in others the nuclei were single. There was evidence that autogamy takes place in this parasite even in an unnatural host. Smear preparations of sporonts showed the characteristic 'pre-metachromatic granule'. Spores appeared the same as the spores in *Moniezia* (Fig. 82,83). They were however smaller in size.

In many of the heavy infections of long duration spores were nearly always confined to the cortical regions of the worm. Whenever the medulla was invaded the membranous partitions of the genital glands were never transgressed.

Although attempts at demonstrating the cuticle-penetrating stage of the parasite in these worms were unsuccessful, it is quite clear that this is indeed the method by which the parasite gains entrance into the tissues of the worm. In support of this is the fact that in all early infections the developmental stages are found in the cortex just under the subcuticular layer.

The demonstration of the possibility of infecting mice and rat tapeworms by feeding spores to these vertebrates, almost certainly proves that a similar condition obtains in nature in *Moniezia* of sheep.

Although numerous sections of the small intestines of mice were cut with worms in situ, no evidence of any infection of the intestinal tissue of the mice with the microsporidian was obtained.

It is seen that the longer the infection in these experimental worms is allowed to run, then the greater is the chance of finding a 100 % infection in them (Experiment 5). The negatives in those worms examined after short periods of infection are probably due to developmental stages only being present.



Finally, the ability to infect these worms of mice and rats suggests that Nosema helminthorum is infective to tapeworms--justifies Moniez's name for this species "helminthorum". As he also believed that the same parasite is found in a nematode of the cat (Toxocara cati), an attempt was made to see whether the oxyurids found in some of the experimental mice would get infected with this organism. Although several of them were exposed to infection none of them were found to become infected.

The infectivity of N. helminthorum to H. nana which belongs to a different family to the Moniezias, suggested that this organism is a potential parasite of other cestodes. It was therefore decided to test its infectivity on several other cestodes.

## 2. Experiments with Cestodes of the Jackdaw

As the common jackdaw was found to be a ready source of infected tapeworms, it was decided to see if N. helminthorum could infect these worms experimentally.

Fifteen jackdaws captured in St. Albans were used in these experiments. Five of them were killed at once

after a preliminary examination of the faeces showed no evidence of helminth ova. Three of these birds had cestodes belonging to the following species:- Hymenolepis sp. and Anomotaenia sp. Eight worms were recovered in all and it was decided to treat them as controls. All these worms were therefore examined in great detail for evidence of naturally occurring infection. For this purpose representative portions of each of the worms were sectioned serially, while the rest of the material was examined by squashing. The results were negative.

Of the ten remaining jackdaws it was decided to feed them all with spores of N. helminthorum. This was done because it was not practicable to find out which of them harboured tapeworms. These birds were therefore ringed and numbered and each was given a different dose of the spores. The spores were obtained in a concentrated emulsion of heavily infected segments of Moniezia by means of a homogeniser. This suspension was fed in varying doses by inserting a narrow catheter-tubing into the gullet of the bird and forcing the material in from an injection syringe.

The details and results are indicated in the table below. It is seen from this table that none of



the seventeen cestodes exposed to infection became infected. The cestodes from these experimental birds belonged to the same species as mentioned above.

Jackdaw	1	2	3	4	5	6	
Quantity	2 c.c.	2.c.c.	2 c.c.	10 c.c.	5 c.c.	2 c.c.	
Date fed	25.8.54	25.8.54	31.8.54	31.8.54	31.8.54	31.8.54	
Date killed	31.8.54	31.8.54	7.9.54	14.9.54	21.9.54	24.9.54	
Duration	6 Days	6 Days	7 Days	14 Days	21 Days	21 Days	
Results	smears } 0/4 sections } - ve	No worms recovered		Only one Acanthocephalan } - ve	0/1 } - ve	0/3 } - ve	No worms recovered

Jackdaw	7	8	9	10
Quantity	2 c.c.	2 c.c.	2 c.c.	5 c.c.
Date fed	-----31. 8. 54 -----			
Date killed	-----21. 9. 54 -----			
Duration	----- 21 Days -----			
Results	Smears } 0/4 sections } - ve	0/5 } - ve		NO WORMS

Discussion

From the above results it appears that Nosema helminthorum will not infect cestodes of the jackdaw. There are two possible explanations for this.

(a) That this parasite is not infective to the two tapeworms of the jackdaw that were exposed to infection.

(b) That the digestive juices of the jackdaw are not suitable for successful emergence of the sporoplasm from the spores of this microsporidian. Even if the sporoplasms do emerge, they do not seem to do so in that portion of the bird's gut where the worms are present, hence there are destroyed before they can infect these worms.

The results of the experiments with H. nana of mice and rats, and Taenia saginata of man suggest that N. helminthorum can infect cestodes belonging to different families. It is therefore more reasonable to regard the failure to infect the cestodes of the jackdaw as due to the unsuitable conditions for emergence and entry of the sporoplasm.



3. Experiments with Taenia saginata of man

Arrangements were made to see whether the Taenias of man could be infected with N. helminthorum under experimental conditions. By kind permission of Professor A.W. Woodruff, and with the help and co-operation of his staff in the Chamberlain Ward of The Hospital for Tropical Diseases, experiments were carried out on two patients admitted to be treated for tapeworm infestation.

Case I- The patient was fed a concentrated emulsion of spores as well as some heavily infected segments kept in the refrigerator for about three weeks. These were administered in gelatin capsules, two capsules being given on the morning of 6.11.54 and two on the evening of the same day. Segments passed by the patient on 6.11.54 were collected for examination as controls. Some segments passed on 10.11.54 were also collected. The vermifuge was given on 11.11.54 and the whole worm, complete with scolex, was passed on the same day.

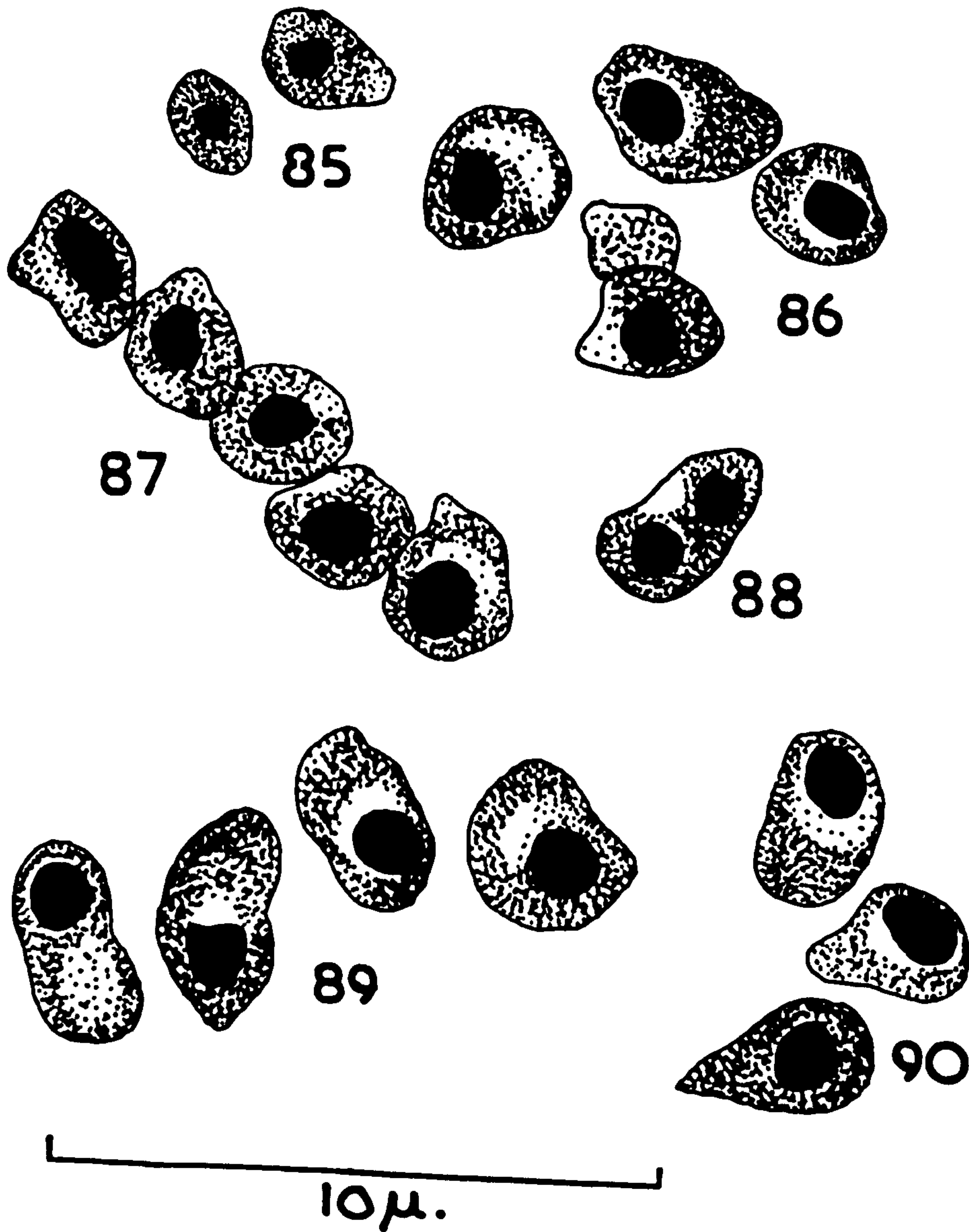
The examination of the scolex and the arrangement of the uterine branches of the gravid segments showed that the worm was Taenia saginata. It was then examined as thoroughly as possible by crushing many of

the numerous segments in saline, and examining smears for evidence of spores or developmental stages . The remaining segments were fixed in Carnoy's Fluid for sectioning. Segments for sectioning were selected from representative regions from twenty different parts of the worm. Serial sections from these regions were cut at 4-5  $\mu$ . thickness and examined thoroughly for developmental stages and spores. The control segments passed on the day of feeding the spores, and the segments passed on the day before treatment were also examined in the same way. In neither of the sections were any signs of infection detected. It was possible, however that the particular spots that had been infected may have been missed.

Case II- The patient was fed a concentrated emulsion of spores and also infected segments of Moniezia on the evening of 5.3.55. These were administered in gelatin capsules. The following morning a concentrated emulsion of spores, obtained by centrifuging a sieved saline extract of the crushed worms, was given in flavoured milk. The patient was treated on 8.3.54 and the worm passed on the same day. Unfortunately in this case no control segments passed on the day of feeding could be collected. As in the first case the



PLATE XIV



Developmental stages from sections  
of Taenia saginata from Case II.



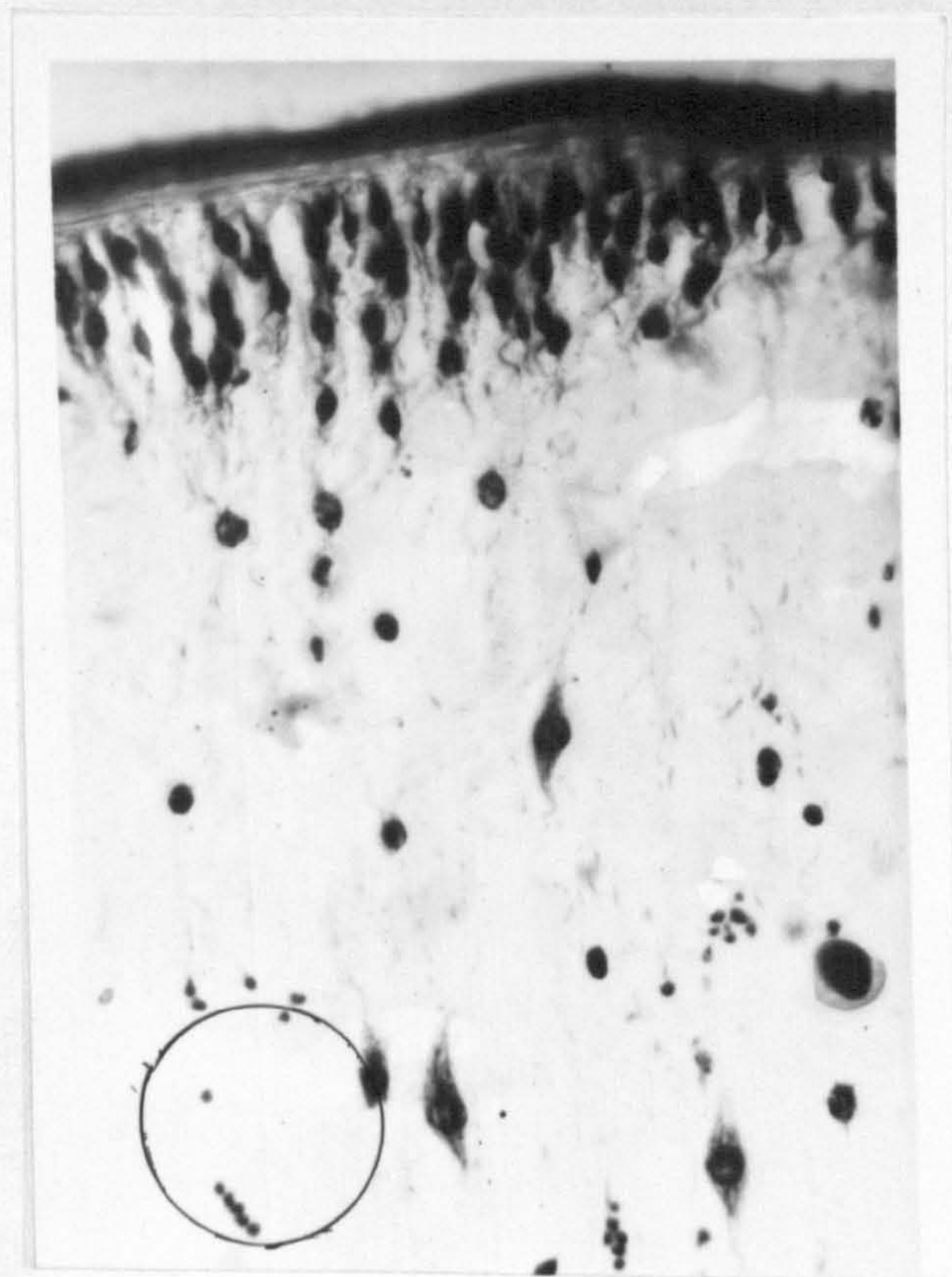


Fig. N.

Transverse section of Taenia saginata from Case II, showing several areas in cortex with developmental stages. X 600 (Giemsa-Colophonium)

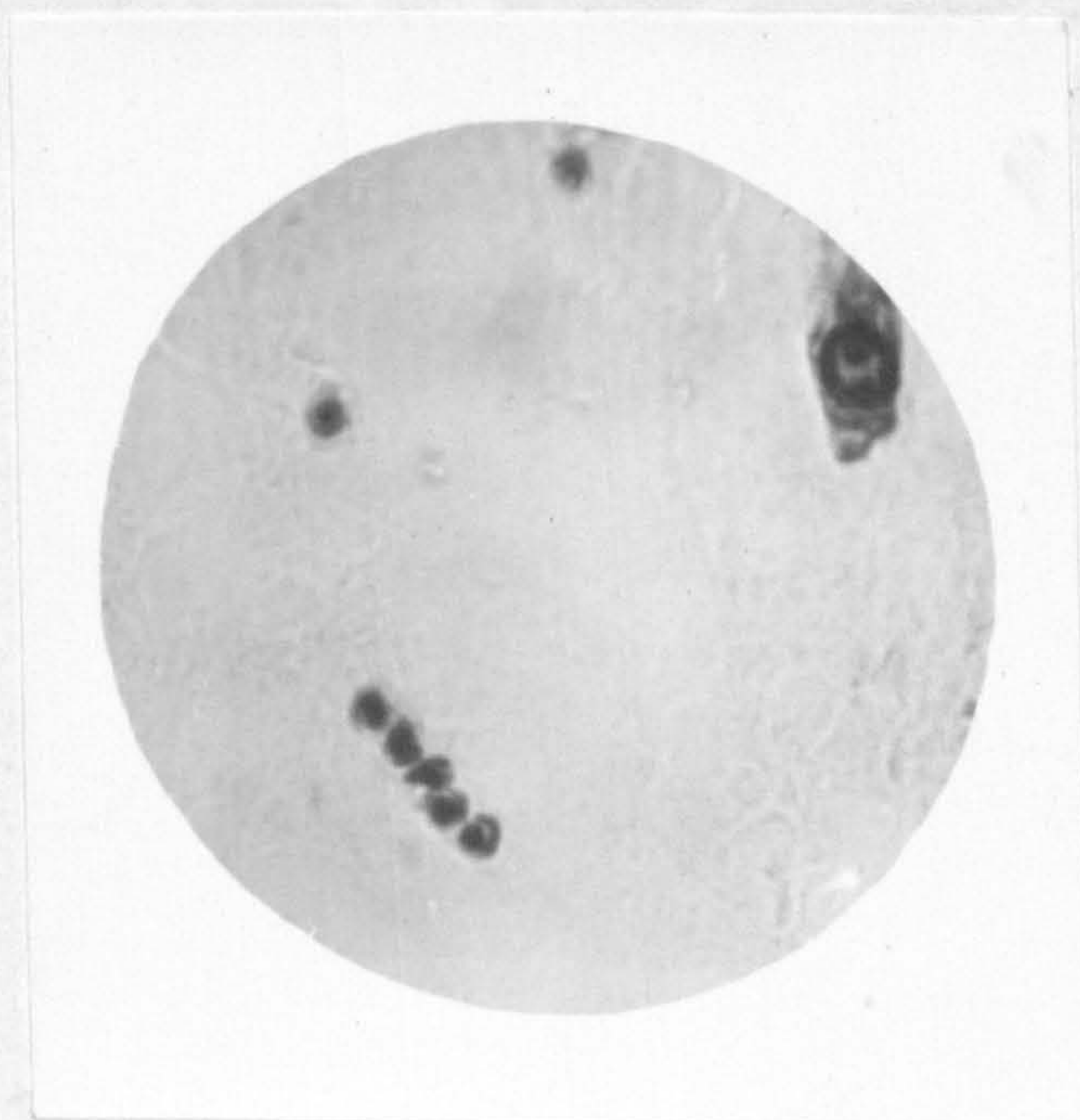


Fig. O.

Ringed area in above section , X 1500



worm was identified as a T. saxinata. A thorough search was made by crushing segments from over a hundred regions, but no spores or vegetative stages could be made out. It must be admitted that vegetative stages are not easy to detect in fresh smears, and as the infection was only of three days duration it was quite possible to miss any early stages by this method. Several segments were fixed in Carnoy's Fluid and segments from eight different regions were sectioned. Sections were cut at  $4\ \mu$  thickness in series. Many segments from mature and gravid regions showed early division stages (Fig. N, O ). (Plate XIV, Fig 87-90 ). These were mostly spherical and between  $1.4 - 2.0\ \mu$  in diameter. The cytoplasm was deeply staining, and the compact nuclei were just visible as red dots in this dark cytoplasm. These stages closely resembled the dividing stages seen in early infections of Moniezia and in experimentally infected H. nana of mice. All these stages were seen in the cortical regions of the worm and were invariably situated at some distance from the cuticle ( about  $100\ \mu$  ). This suggested that the original sporoplasms had travelled a fair distance inwards before commencing to divide. In several places chains of schizonts were seen ( Plate XIV, Fig. 87, and Fig. N, O ). These were probably formed by a series of binary fissions.

It is possible, however, that an elongate multinucleate schizont gives rise to this effect. The short duration of the infection accounts for the absence of spores or even sporont stages in sections. This experiment shows that the number of days required for spore formation is over three. It was not practicable to keep a patient for such a long period before treating him. It was decided in future to try to feed the spores to the patient in the out-patients department, thus by the time he entered hospital for treatment the infection could have then taken place long enough to produce spores. This was a necessary step to prove conclusively that the stages seen in the worm from the second patient were those of a microsporidian. There was little doubt, however, that they resembled the typical early stages of H. helminthorum in Moniezias and in H. nana of mice and rats. Unfortunately time did not permit this experiment to be carried out.



4. Experiments with Taenia hydatigena of the dog.

It was next decided to feed the spores of the protozoan to a dog already infected with a tapeworm. Accordingly a dog was fed four cysticerci from a sheep (Cysticercus tenuicollis) on 25.11.54. The faeces of this dog were then examined from time to time after about three weeks. They were negative on many occasions until on 18.2.55 by using the concentration method, several eggs were detected in the faeces. This showed that the infection with T. hydatigena had taken place and that the worms were mature. On 25.2.55 this dog was fed a very large quantity of spores in about twenty-five heavily infected segments of M. expansa. On 11.3.55 another lot of spores was fed and at the same time some segments that had been passed by the dog on 9.3.55 were examined by crushing and by sectioning, for evidence of infection, but with negative results.

On 25.4.55 the dog was prepared for worming by starving it after the morning meal. On the following afternoon it was given 60 minims of Liquid Extract of Male Fern in capsules. The following day a large number of segments were passed, and then a dose of magnesium sulphate was given. After this a few more segments were

evacuated. It was not possible to say how many worms were passed as the scolices could not be recovered from any of them. The segments that were passed were divided into two lots, one lot was fixed in Carnoy's Fluid for sectioning and the other was placed in normal saline for examining fresh smears of the crushed segments.

Over 200 segments examined by crushing in saline showed no evidence of any spores or of developmental stages of <sup>the</sup> microsporidian. It was therefore decided to make sections of the Carnoy-Fixed material for a more detailed examination for various stages of the parasite. Sections were made from twelve different regions and examined in very great detail. No evidence of either spores or developmental stages were noted. Sections from ten more segments from various regions were made, but again no stage of the parasite was seen.

### Discussion

Although many regions of the worms must have escaped examination it is highly improbable that even if this examination was done more thoroughly the parasite would have been found. For one thing the infection would have gone on for two months since the first feed with spores, and this would have given ample



time for spores to be detected in several regions of the worm. It is therefore concluded that it was not possible to infect T. hydatigena of the dog with N. helminthorum in this one experiment. It is possible though unlikely, that the infection could have begun with developmental stages as in the case of T. saginata, but that these were abortive and soon disappeared.

##### 5. Experiments with Rabbit Cestodes.

It was thought necessary to try to infect Cittotaenia of wild rabbits with N. helminthorum particularly because this genus too belongs to the family Anoplocephalidae. Unfortunately due to the myxomatosis epidemic it was not possible to perform any experiments that were desired. Recently, however a few wild rabbits tested and found negative for myxomatosis, were made available to me. But none of these animals had any cestodes in them and so these experiments had to be abandoned.

Chapter VII

Distribution of Nosema helminthorum

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(a) Host distribution

Nosema helminthorum has been found in the present work to be hyperparasitic in the following anoplocephalid cestodes, Moniezia expansa and M. benedeni of sheep in this country, and Moniezia sp. from a buffalo calf in Pakistan. Moniez (1887) regarded the microsporidian he reported from Ascaris mystax (= Toxocara cati) of the cat as identical with the parasite he found in M. expansa and M. benedeni, and Labbé (1889) said it was the same parasite he found in T. expansa (= M. expansa), T. denticulata (= M. benedeni), T. bacillaris (= Hymenolepis bacillaris) and in Toxocara cati though he called it Plistophora helminthophthora. Skrjabin (1923, 1927) reported N. helminthorum from Toxascaris limbata of the dog. Whether the parasites found by Moniez, Labbé, and by Skrjabin in the nematodes of dogs and cats are the same as N. helminthorum it is difficult to decide, unless future experimental infection of these nematodes with this organism are made. The microsporidian discovered in Ostertagia circumcincta may also probably be Nosema



helminthorum, and in this instance it is much more likely to be the case as the main host in this too is sheep. However it is only by experimental demonstration of the fact that N. helminthorum can infect Ostertagia, producing sterility in the females, that this possibility can be accepted at all seriously.

In view of the fact that Cittotaenia of wild rabbits is an anoplocephalid cestode like Moniezia and in view of the common habitats and intermediate hosts which sheep and rabbits share it is rather surprising that this parasite was <sup>not</sup> found occurring naturally in the rabbit tapeworms. It is unfortunate that this could not be tested experimentally. But as will be pointed out in the chapter on host restriction, it is quite possible that these worms are in some way refractory to this microsporidian.

As discussed earlier the ability of N. helminthorum to infect a wide variety of host tapeworms under experimental conditions, and even certain oribatid mites, suggests that this organism can under suitable conditions infect many tapeworms provided that the conditions for emergence of the sporoplasm and its 'application' to the host tissue are satisfactory. Its limited distribution in only the Moniezias in nature may well be due to the



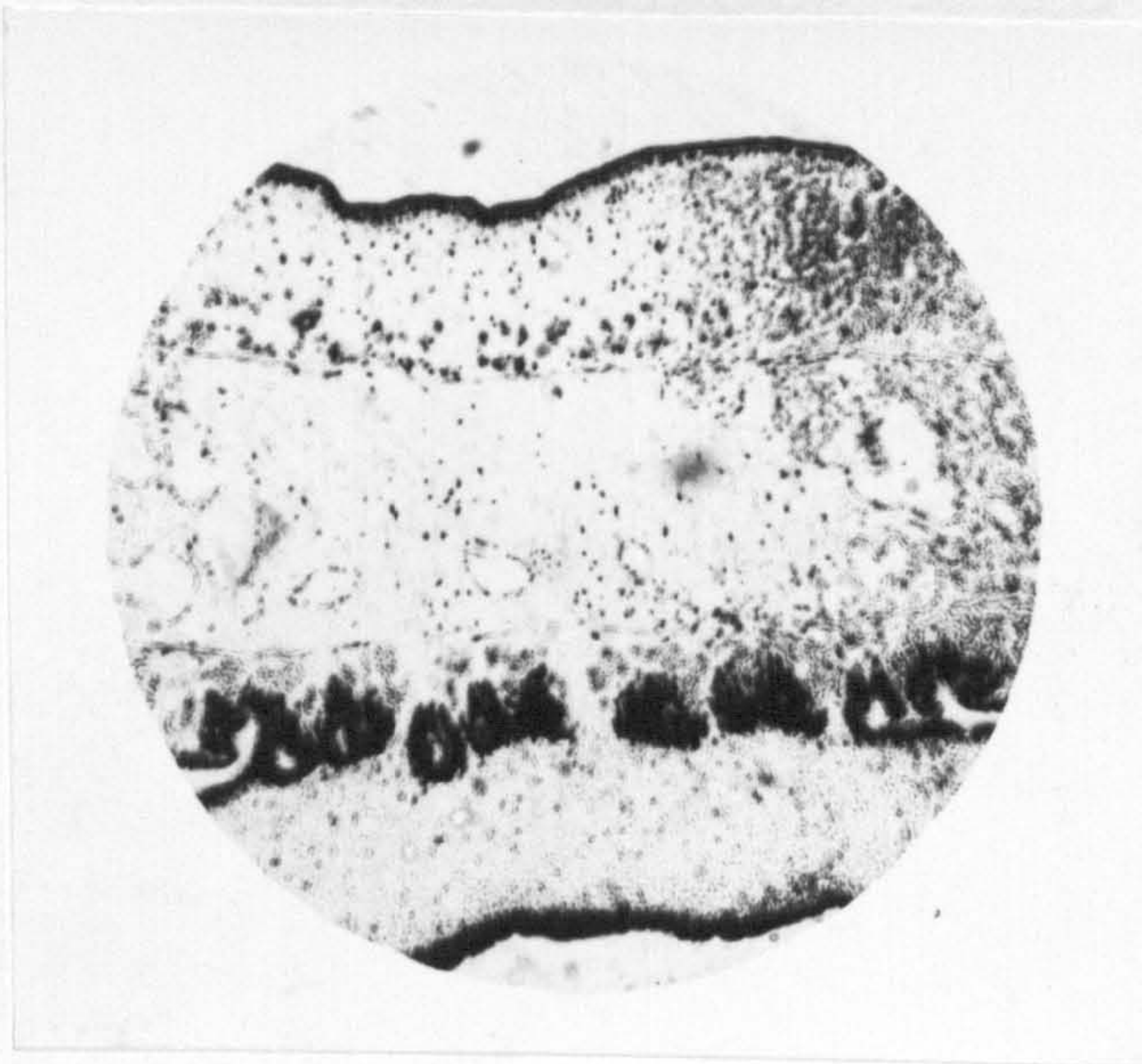


Fig. P.  
Section of M.expansa  
showing infection , with  
spores confined to cortex  
in one area. ( G.C.)

( X 65 )

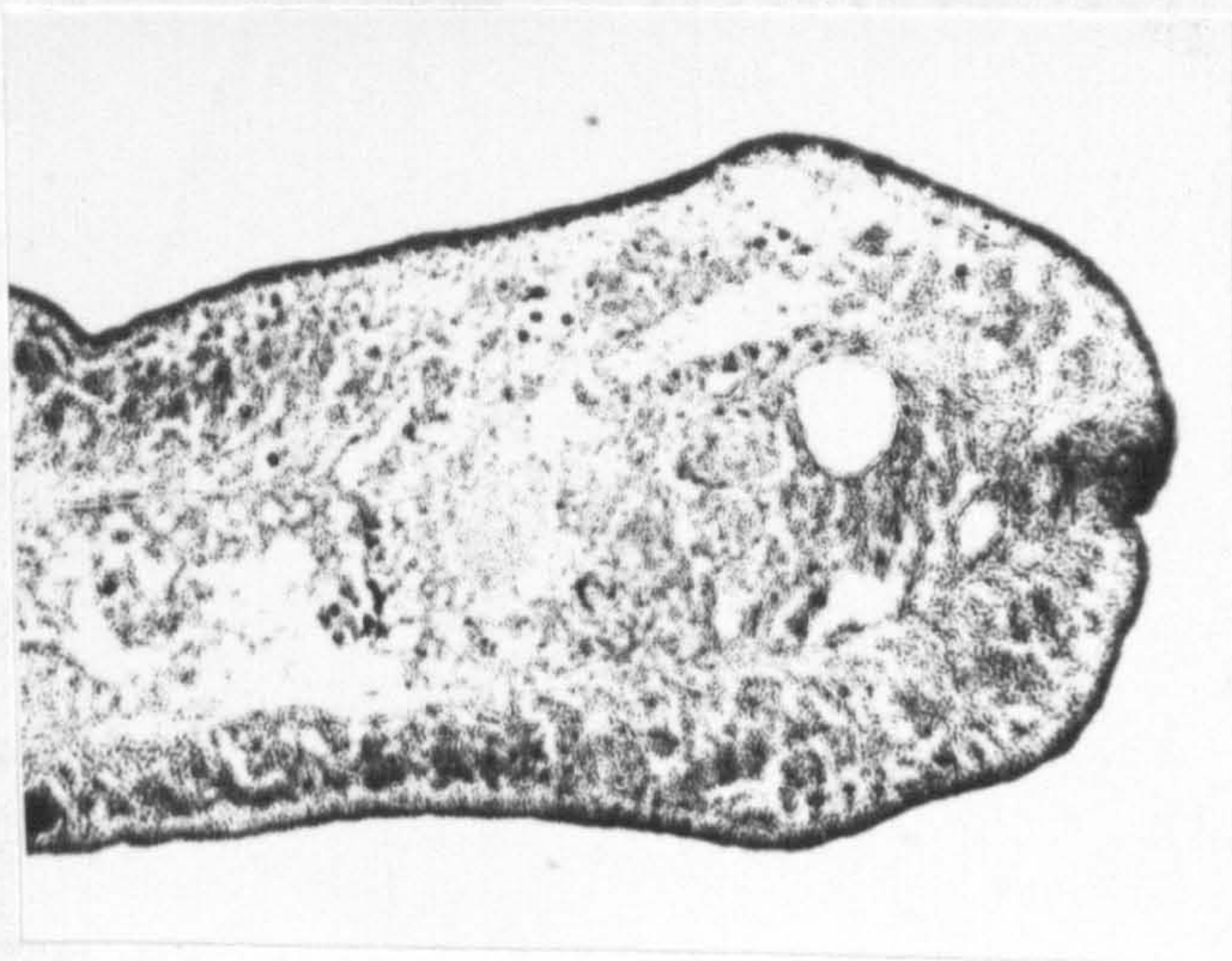


Fig. Q.  
Heavily infected worm in  
T.S. showing spores in  
medulla as well.(G.C.)

( X 65 )



Fig. R.  
T.S. of a heavily infected  
worm showing destruction  
of tissue and infiltration  
of genital glands.(G.C)

( X 85 )



fact that, among other things, the other tapeworms do not come into proper relations with the parasite owing to the different habitats of their vertebrate hosts.

As a rule most microsporidia "are found in specific situations in a single host species" (Kudo 1924). There are however a few exceptions to this rule. For instance Nosema bombycis attacks every tissue of all developmental stages of the silk-worm, Bombyx mori. In cases of hyperparasitism, the microsporidia rarely attack the main vertebrate host although one example of this is found in Glugea danilewskyi which attacks both the vertebrate host and the trematode living in one of them. We have seen that in Nosema helminthorum although the infection is generally confined to the more posterior and gravid segments no region of these tapeworms is resistant to the infection. This is well illustrated in U 93 where the scolex and anterior segments were infected while the posterior segments were quite free of infection. At the same time the portion of the tapeworm tissue that is generally most affected is the cortical region (fig. P,Q,R) and rarely is the medulla attacked, yet in very heavy infections the membranous partitions of the genitalia are transgressed by the parasite (fig. R.)<sup>and</sup> developmental stages and spores may

be seen in these organs. In such instances the ova in the gravid segments do not seem to be able to give rise to normal embryonated eggs, and so the possibility of transovarian infection must be overruled. In two cases where the intestines from which the infected *Moniezia*s were recovered, sections of the intestines showed no evidence of infection with the microsporidian, while a large number of mice-intestines containing experimentally infected Hymenolepis nana were sectioned but no trace of any stages of the microsporidian were seen even here. All this proves that the vertebrate host is unaffected by the microsporidian in the tapeworms.

(b) Geographical Distribution

The wide distribution of a microsporidian in different localities is not an uncommon feature of these protozoa. Nosema apis, for instance, has been found in Australia, Canada, Denmark, Germany, Gt. Britain, Natal, Switzerland, and U. S. A, while Nosema bombycis has been reported from Australia, Brazil, France, Germany, India, Italy and Japan. It would appear from these that the parasite is present in any country in which the host occurs.

Moniezia and other anoplocephalid cestodes of ruminants are almost world-wide in distribution, and it



is therefore logical to expect that the microsporidian is also similarly distributed. Moniez (1879, 1887) described the parasite from France; Labbé (1899) from Germany, and more recently Weiser (1951) from Yugoslavia. I have found the same parasite from Great Britain and Pakistan, and felt that an examination of worms from several other countries would be of interest. Accordingly I obtained as many specimens of Moniezia and other anoplocephalids like Helicometra from Africa, Australia, and Ceylon and a few other countries. The African material was made available to me by Mr. S. Prudhoe of the British Museum (Natural History) who gave me many serial sections and a few segments from Moniezias in the collection, to examine for evidence of infection. No infection with this microsporidian was detected in any of the worms examined belonging to the following species, Moniezia expansa, M. benedeni and M. mattami

A few more specimens from Africa were given me by Dr. P. L. Le Roux, but none of these showed any infection either.

The results of these examinations are summarised on the following page.

<u>Segments examined</u>	<u>Host</u>	<u>Locality</u>	<u>Result</u>
1. <u>M. expansa</u>	Goat	Uganda	-ve
2. <u>M. expansa</u>	Sheep	Deccan	-ve
3. <u>M. expansa</u>	Reindeer	Spitzbergen	-ve
4. <u>M. benedeni</u>	Bull	Uganda	-ve
5. <u>M. benedeni</u>	Angora goat	Uganda	-ve
6. <u>M. Voniezia</u> sp.	Camel		-ve
7. <u>M. Voniezia</u> sp.	Cattle	Sudan	-ve

<u>Sections examined</u>			
1. <u>M. mattami</u>	Warthog	Uganda	-ve
2. <u>M. alba</u>	?	Rangoon	-ve
3. <u>M. benedeni</u>	Solan Reed buck	Tanganyika	-ve



Several specimens of anoplocephalid cestodes of sheep were kindly sent to me by Mr. H. McL. Gordon of the McMaster Animal Health Laboratory, N. S. Wales, Australia. These worms were sent preserved in 5 % Formalin. They belonged to the two genera Moniezia and Helicometra. None of the 25 complete worms in which the scolices were intact, nor any of the large number of broken segments examined in very great detail by crushing showed any evidence of microsporidian spores. As there were no positives among these worms no attempt was made to identify the species of these worms.

A batch of anoplocephalids from goats in Colombo, Ceylon were sent to me by Mr. C. de S. Kulasiri of the Medical Research Institute, Colombo. There were in this collection( which was also preserved in 5% Formalin) 29 complete specimens and several broken segments belonging to the two species of Moniezia expansa and M. benedeni . Although once again a thorough search was made by examining the crushed segments of these worms no spores were seen.

It is obvious from these results that the number of worms examined from the different countries was insufficient to make a definite statement as to the presence or absence of N. helminthorum in them. While

even a single positive from a country proves the existence of this parasite in that region, unless several hundreds of worms from different localities in a country are found to be negative one cannot state definitely that the parasite does not occur there. As mentioned before, the fact that the host tapeworms are found in almost all parts of the world, and also the known reports of M. helminthorum from such countries as Gt. Britain, France, Germany, Yugoslavia and Pakistan lead one to the conclusion that this parasite will eventually be found in all regions where the corresponding anoplocephalids are present. Where other genera than Moniezia are prevalent in a country (in sheep, goats and cattle and even other ruminants), there is no reason why this parasite should not be found in these genera. It is quite possible however that, as has been shown by Canning (1955) for Nosema locustae, the parasite occurring in the different geographical regions may not be the same strain and although it has the same morphological features it may be a different biological race that one comes across in the various regions.



## Chapter VIII

### Host-Parasite Relationships

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#### (a) Effects of the microsporidian on the tapeworm.

##### Macroscopic changes

As was mentioned earlier in the general account of the infection in *Moniezias*, the majority of worms show no externally visible signs of infection with this microsporidian, nor do they differ in their behaviour from the normal worms even when heavily parasitised. There occur, however, in a few worms as seen before, white opaque areas of infection which can readily be recognized with the naked eye (fig. A). In these the spores are so heavily concentrated in localised spots in the worm that they give the appearance of miliary tubercles. The white spots are generally not more than 1-2 mm. in diameter and are either circular in outline or more or less square. They are scattered anywhere in the length of the worm with no preference to a particular region in the segment, as was pointed out by Weiser (1851) who found that these areas were more or less confined to the posterior regions of the segments. Many of these areas of infection are seen to extend

from the posterior end of one segment to the anterior portion of the next.

Microscopic changes

Before describing the microscopic effects of this parasite on the host tapeworms it is necessary to give a brief description of the internal structure of these worms as seen in sections. Outermost is a thick cuticle. This rests on a structureless basement membrane to which are attached the ends of the sub-cuticular cells which are believed to be the sunken epidermal cells in these worms. Amongst these sub-cuticular cells are two layers of muscle, an outer circular and an inner longitudinal. Between the above structures and the medullary region of the worms, which contains the genital glands, is the cortex which is filled up with loose mesenchymatous tissue generally referred to as the parenchyma of the worm and in which are embedded the muscle bundles, nervous and excretory network. Separating the cortex from the medulla is an outer well developed layer of longitudinal muscle bundles and a layer of circular muscle fibres which is less prominent. The loose parenchymatous tissue which fills all the space in the cortex and the medulla of these tapeworms is of great importance in these infections.



It is unfortunate therefore that the histological structure of this tissue is obscure. All the workers agree that this consists of a faintly staining network or ground substance which encloses polyhedral spaces, but as to the exact details of its structure there is much controversy. According to some, these spaces are intracellular and formed by the coherence of bladder-like cells. Another group of observers believe that these spaces are inter-cellular and formed by the meeting and fusion of the branching processes of embryonic cells. It is possible however that the parenchyma is formed in both these ways as pointed out by Wisniewski (1930).

Scattered through the parenchyma are relatively large round or oval nuclei which are presumably nuclei of sunken sub-cuticular cells. Also found in the parenchyma though not so well developed in *Moniezia* are the calcareous corpuscles.

The various stages of Nosema helminthorum are found in the parenchyma of Moniezia, and depending on the stage of the infection these are present either close to the sub-cuticular cells or may spread deeper into the cortex and even into the medulla. None of the characteristic effects that microsporidia generally produce in their hosts are seen in tapeworms. For

instance, nuclear and cell hypertrophy are not observed, nor is there any evidence of a phagocytic reaction on the part of the host. The only effect of the parasite is the destruction of host tissue by infiltration and replacement. This is most marked in places where the development of the parasite has gone to the spore stage, when masses of spores are found to be present in areas completely devoid of host tissue (Fig. Q, R ). In such areas groups of spores may be seen (in sections) to be enveloped within cysts which are actually formed by the surrounding host tissue.

Most of the microsporidia that have hitherto been described are intracellular, with the single exception of Nosema binucleatum described by Weissenberg (1926). This organism is found in the intercellular spaces of the larvae of Tipula gigantea. It is difficult to decide whether Nosema helminthorum is intracellular or intercellular owing to the uncertainty that exists regarding the nature of the parenchyma. All the developmental stages, seen whether in naturally infected Moniezias or experimentally infected Hymenolepis nana and Taenia saginata, are clearly within the faintly staining ground substance of the parenchyma and not in the actual spaces. Spores on



the other hand, may in some instances be seen free in these spaces, and sometimes the spaces appear much larger due to the destruction of surrounding tissues. The fusiform schizonts seen in M125 and M135 are interesting in that they are clearly in the actual substance of the parenchyma and when they happen to be adjacent to one of these spaces they take on a curved appearance and partially surround the space. If it is the rule that microsporidia develop intracellularly then it would have been possible to say that the ground substance of the parenchyma of cestodes is really intracellular material but the case of Nosema binucleatum shows that some caution must be exercised in making such statements.

Jones (1943) found that Stempellia moniezia, which he described from two cestodes of an insectivore, were more or less confined to the region surrounding the excretory canals. Weiser (1951) found that there was a tendency for the parasite to be concentrated in the posterior edge of a segment. None of these observations were confirmed in the present case. It was found that the parasite could occur anywhere in the segment depending on where the initial infection took place.

Pathogenicity

From my observations on the many naturally infected *Moniezia* and the experimentally infected *Hymenolepis nana*, it is clear that *N. helminthorum* is not pathogenic to its host. Apart from the replacement and destruction of tissue (Fig. Q, R) this parasite causes no other adverse effects, and the fact that cestodes do not possess any vital organs shows that even if the entire tapeworm tissue were eventually attacked by the parasite it would cause no serious harm. For one thing the fact that the genital organs are rarely if ever attacked, minimises the possibility of sterility. Furthermore, as is seen in most of the parasitised worms, the more posterior segments seem to be ones generally involved, and these being gravid at the time, are soon sloughed off so that there would be no time for the infection to spread to the genital glands.



(b). Host-range and Host-restriction

As Nosema helminthorum was found to be infective to Hymenolepis nana of mice it was decided to see whether other sporozoan spores could be made to infect these worms experimentally. Accordingly spores of Nosema locustae a microsporidian from a locust, and the oocysts of a schizogregarine Tribolilocystis garhhami Diasanaike, (in press) were employed in a few experiments which will now be described.

1. Attempts at infecting Hymenolepis nana with spores of Nosema locustae.

Two experiments were performed. In the first of these, one mouse known to harbour H. nana was fed spores of N. locustae from a heavily infected locust, while another mouse was kept as a control. The spores were kindly given to me by Miss Canning who was at the time working on this parasite.

The spores were fed on 26.7.54 and the mouse sacrificed on 4.8.54, 9 days after. None of 10 worms examined by crushing showed any infection, while the same was true of nine worms stained by Newton's method. Of the four worms recovered from the control mouse none showed any infection.

In the second experiment, two mice were fed with spores of N. locustae on 10.8.54. One mouse was used as a control. One of the experimental mice was sacrificed on 19.8.54 nine days after the feed. None of the sixteen worms examined by crushing and by Newton's method showed any signs of infection. The second mouse was killed on 26.8.54, sixteen days after the feed and none of four worms recovered from it showed any infection. Five worms were recovered from the control mouse but were all negative.

From these experiments it is concluded that N. locustae is not infective to H. nana of mice.

2. Attempts to infect H. nana of mice with oöcysts of a schizogregarine Tribollocystis garnhami

These oöcysts were available in large numbers from the infected larvae of a culture of Tribolium castaneum and were fed to two mice on 7.12.54, two other mice being kept as controls. One of the experimental mice was killed on 17.12.54, ten days after the feed, while the other was killed on 22.12.54 fifteen days after. The results of the examination of worms from the two mice are as follows :-



Mouse I	Smears	0/7	Mouse II	Smears	0/3
	Newton's	0/10		Newton's	0/7
	Sections	0/3.		Sections	0/2

None of the seven worms recovered from each of the two control mice showed any infection.

Thus none of the thirty-two worms exposed to infection became infected. The conclusion is that this schizogregarine is not infective to H. nana.

#### Significance of the experimental work

The fact that H. nana of mice and rats and T. saginata of man could be infected with the spores of Nosema helminthorum shows that this microsporidian of Moniezia has a high infectivity for cestodes of different families. The experiments carried out with spores of N. locustae and oöcysts of Tribolilocystis garnhami were performed because this material was available in large quantities, and the negative results only proved what was to be expected, that these organisms are restricted to the

corresponding arthropods, and are not infective to tapeworms.

As for N. helminthorum itself, it is clear from the fact that this organism can infect R. nana of mice and rats as well as T. saginata of man, that the failure to infect a number of other tapeworms was due to one or more of several reasons: -

(a) The infection probably 'took' in some of them but the methods of examination were such that certain parts of the worms could have been missed, and the infection may have been present in these particular parts.

(b) The conditions in the digestive tracts of vertebrate hosts in which the tapeworms failed to become infected were unsuitable for the successful emergence of the sporoplasms from the spores. Even if this did occur it happened in that part of the digestive tract where the worms were not present.

(c) The particular worms that failed to become infected were not susceptible to infection with this microsporidian, or the microsporidian was not infective to these same cestodes.

There is little doubt that the first of the above explanations is unlikely for the simple reason that many



thousands of spores were fed in each case and it is probable that many scattered parts of the worms were infected so that at least one of these areas should have been spotted in the series of sections from different parts. In the case of the dog tapeworms, the duration of the infection, if it 'took', was sufficient for mature spores to have been present in large numbers in many regions. The failure to find any infection in these worms showed that the infection had not 'taken'. The same was true of the cestodes of the jackdaws. The failure to infect the tapeworm in the first human case, even though the duration was greater than in the second case, shows that infected areas in the worm from the first case may have been missed, or, more probably, that early stages were present as in the second case but that they soon degenerated as conditions in T. saginata are unsuitable for further development of the parasite. The other possibility, of course, is the conditions for successful emergence of the sporoplasm and entry into the tapeworm tissue were not present in the intestinal tract of the first patient.

The fact that it was possible to infect two oribatid mites X. tegeocranus and Ceratoppia bipilis, shows that the infectivity of this microsporidian is not confined to the cestodes.

Discussion : Host-restriction.

Hegner(1926) defines host-parasite-specificity as "the character of the relationship between species of parasite and species of host with respect to host susceptibility and parasite infectivity". While it is true that the range of hosts attacked by a given parasite depends on these two factors, it must be pointed out that the term host-parasite-specificity is incorrect, as shown by Hertig, Taliaferro and Schwartz(1937). They stated " it is obvious that there may be host-specificity on the part of the parasite, but it can hardly be maintained that the converse exists namely parasite-specificity on the part of a given host ". Becker (1933a) distinguishes between specificity of a parasite by which is meant the quality of distinctness from all other parasites, and host-specificity which may be rigid or loose according as the parasite confines or fails to confine its host selection to a certain group of animals. It is well known that in practical considerations of speciation, specificity as defined by Becker and host-specificity are in some ways connected. Hoare (1943) has therefore preferred the term host-restriction to host-specificity " in view of the different connotations of the latter term in biology. " The term host restriction was used by Culbertson(1941) who considered all parasites to have either a restricted or a wide host range according to



whether a few or several hosts were attacked. It is clear from this that the term host-specificity should be avoided, and it is therefore proposed to use the terms host-range and host-restriction instead, in the subsequent discussion.

If we consider N. helminthorum from the point of view of host-range and host-restriction we find that in nature, as far as we are aware, the parasite is confined to the Moniezias of sheep and cattle. Under experimental conditions it has been possible to infect Hymenolepis nana (Hymenolepididae) of mice and rats, Taenia saginata (Taeniidae) of man, while attempts to infect T. hydatigena of the dog, and Hymenolepis sp. and Anomotaenia sp. of the jackdaw were unsuccessful. This shows that cestodes belonging to the widely different families Taeniidae, Hymenolepididae and Anoplocephalidae are susceptible to infection with this microsporidian although a Taenia of the dog and a Hymenolepis of the jackdaw were refractory. While it is true that the number of experiments performed with the dog-tapeworms was too small to make any such statement, it is clear that the susceptibility to infection with this parasite certainly does not depend on the group to which the cestodes belong. My opinion is that N. helminthorum is probably infective to cestodes

in general. However, factors in the vertebrate hosts favouring successful emergence of the sporoplasm followed by its entry into the tapeworm tissue, are more important in deciding whether the worms can be infected or not. It may also be that some other factor in the individual tapeworms such as incompatibility of the protoplasm may account for the failure of the infection to 'take' in some worms.

Very little work has been done on the question of host-range and host-restriction in the microsporidia. A few of the known forms have been experimentally introduced into closely related artificial hosts, as in the case of Nosema apis. Kudo (1924, 1924 a and 1925) has called attention to the fact that microsporidian parasites of mosquito larvae are specific to the genus of the host. He has shown that Thelohania legeri, T. obesa, and Nosema anophelis are exclusive parasites of Anopheles, while Thelohania opacita, T. rotunda, T. minuta and Stempellia magna are exclusive parasites of the genus Culex. He found that even when the larvae of these two genera of mosquitoes are found in the same habitat, the infection in them is due to the parasite characteristic of the genus. It is evident from the results of the present work that there is no such generic host-restriction on the part of H. helminthorum and, in fact, the tapeworms infected belonged to widely different families. This,



together with the fact that two oribatid mites were infected with this microsporidian, prompts one to conclude with Becker (1933 a) that "A particular parasite will develop normally in as many hosts as provide for it adequate environmental conditions and mode of entrance."

## Chapter IX

### Conclusions and Summary

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#### Life Cycle of *Nosema helminthorum*

As the life cycle of Moniezia involves two hosts, an oribatid mite and a sheep, it was natural to expect that the life cycle of N. helminthorum would be complicated owing to the possibility of the mite playing some part in the transmission of the microsporidian from one Moniezia to another. As stated earlier in this thesis there are two possible methods of transmission of the microsporidian. In the first, the microsporidian may have a phase of development in the mite. Owing to the fact that the larval stages of *Moniezias* develop in the mite there is a possibility of transference of such an infection in the mite to the tapeworm during its mite-phase. The second possibility is less complicated, and according to it the lamb already infected with Moniezia swallows the spores of the microsporidian which are found contaminating the grass in the grazing ground. In this way the Moniezia gets infected. The best method of testing out these two possibilities would have been to infect a very large number of oribatid mites with Moniezia a larvae, and when the latter are infective, to feed the mites to clean susceptible



lambs. In this way Moniezia-infected lambs would have been obtained and the Moniezias known to be free of microsporidian infection. These lambs could then have been divided into two lots, one fed on spores of N. helminthorum and the other lot kept as controls. If the Moniezias in the first lot became infected, then the simpler of the two possibilities would have been proved. To test the other possibility it would have been necessary to infect oribatid mites with larvae of Moniezia alone, and some with both larvae of the cestode as well as the microsporidian. The mites could then have been examined to see if the larvae had acquired the microsporidian infection from the mites. This could have been followed by feeding both lots of mites to susceptible lambs that were known to be free of Moniezia infection. In this way the role of the oribatid mite could have been tested.

Unfortunately the life history of Moniezia takes several months for its completion. Therefore it was not practicable to carry out any of these experiments. An attempt was, however, made to obtain lambs already infected with Moniezia and to feed them with spores of N. helminthorum with the idea of testing out the simpler of the above possibilities. No success was met with and on one occasion two lambs were spotted that were passing segments of Moniezia in their faeces, but unfortunately these segments

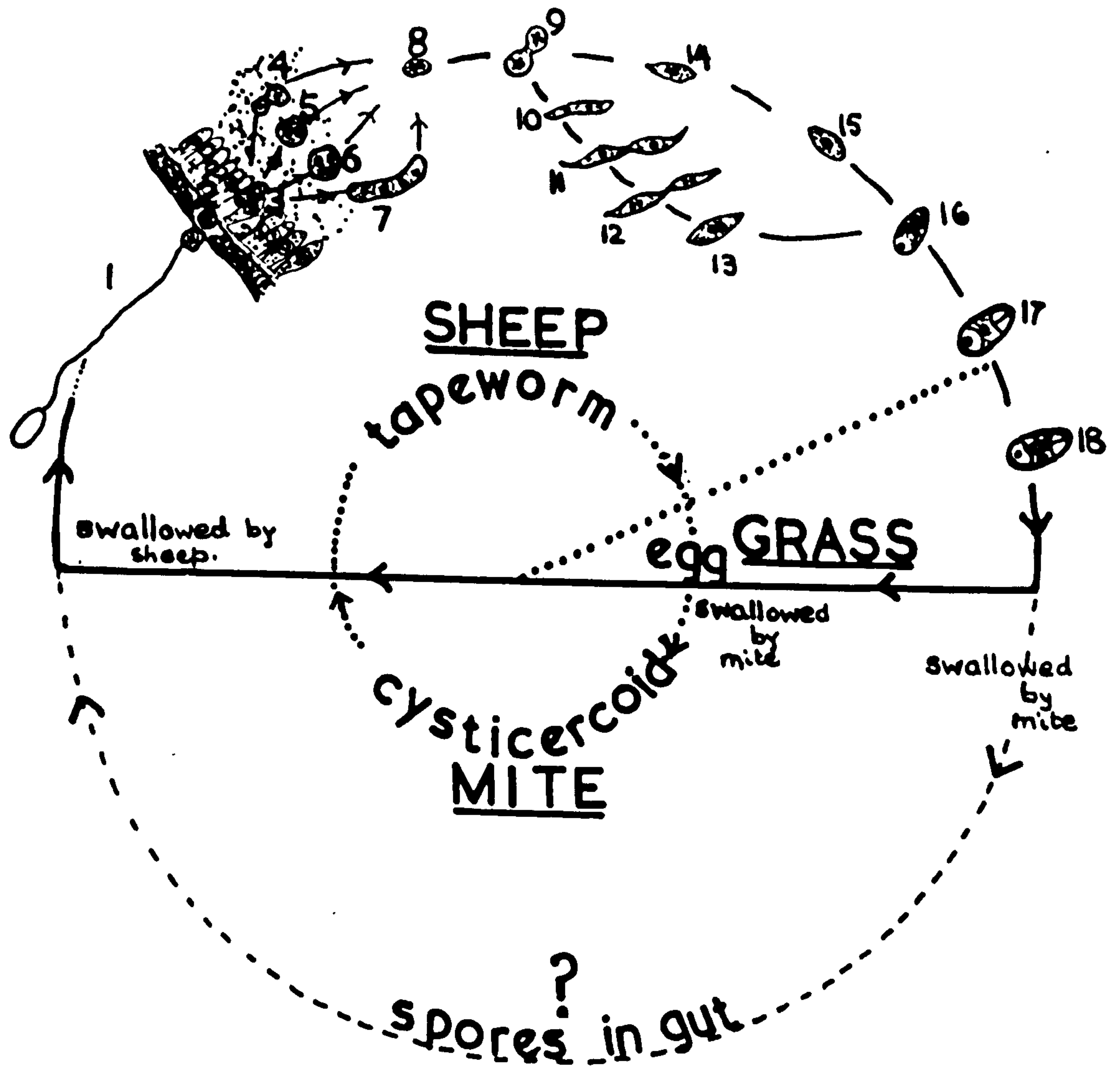
were found to be already infected with the microsporidian.

Although therefore, no experiments on sheep could be performed, the results of the experiments on tapeworms of other animals indicate that the life cycle of Nosema helminthorum is as represented in Plate XV- There is no doubt that the method of transmission is by a sheep already harbouring Moniezia, swallowing spores. Whether spores swallowed by mites and carried through the digestive tract of sheep and protected within the mite, also infect these tapeworms in nature is difficult to decide, but it is not likely.

The spores swallowed by the vertebrate host reach the small intestine, where the tapeworm is already present. Here, some of them extrude their filaments with the sporoplasms attached to their tips. Some of the sporoplasms come in contact with the cuticle of the tapeworm at various places (1). They round off and work their way into the tissues of the worm passing through the cuticle (2). They next proceed between the sub-cuticular cells (3), and from now on start multiplying by binary fission or by multiple division. (4,5,6,7). Some of these so called schizonts are spherical with three to four nuclei (6), while others are elongate plasmodia which give rise to chains of daughter schizonts by division. All these schizonts give rise eventually to faintly-staining schizonts in which



# PLATE XV



Diagrammatic representation of life-cycle  
of Nosema helminthorum

the nuclei appear less compact. Some of these now enter the second phase of schizogony in which ultimately elongate fusiform or spindle shaped cells are produced (9,10,14). The nuclei of these elongate cells divide, and move to opposite poles and the cytoplasm constricts between them. In this way a chain of fusiform schizonts may be formed(11). Eventually the products of these divisions are fusiform or spindle shaped cells (13,15) which are really the precursors of sporonts. In them the nuclei are divided but not separated. These nuclei remain closely associated and eventually fuse either before the sporont stage is reached or later on. The sporonts are ovoidal cells which are generally uninuclear after fusion of the double nuclei of the previous stage. The nucleus is at the centre. Soon a vacuole appears at one pole and within it there appears a pre-metachromatic granule(16), which gives rise to the metachromatic body in the mature spore. A spore wall of chitin is now secreted (17) and the filament is probably formed at this stage being formed in the central axis of the cytoplasm. The rest of the cytoplasm transforms into the sporoplasm which surrounds the filament like a girdle (18). The spore now becomes mature and is infective to a new host. It is passed out in the gravid segments of the worm and when it reaches the exterior it is liberated when the segments degenerate. These spores are quite resistant



and probably tide over the winter and are swallowed by a Moniezia-infected lamb in Spring.

Although Moniez (1887) believed that transmission of this organism takes place through the eggs of the worm in the same manner that has been suggested for Nosema bombycis of the silkworm, I have found no evidence for this method of transmission.

#### Summary

1. The microsporidian found in Moniezia expansa and Moniezia benedeni of sheep in this country, and also Moniezia sp. from a buffalo calf in Pakistan, has been found to be identical with Nosema helminthorum described by Moniez (1887), Plistophora helminthophthora Labbé, 1899, and Nosema bischoffi Weiser, 1951. Therefore the valid name for it is Nosema helminthorum Moniez, 1887.
2. The various developmental stages of this parasite have been described in detail in the present work. Many of the stages have not hitherto been seen by the earlier workers. There is evidence to show that autogamy takes place in the development of this parasite during the second phase of schizogony.
3. Certain fusiform cells met with in some of the infected worms have been shown to be second-phase schizonts with

much drawn out ends.

4. The sporoplasm of the spore of this microsporidian has been shown to emerge in a manner different to that described for other microsporidia. The sporoplasm is attached to the free end of the filament and gets 'whipped out' with it when the filament is extruded.
5. The mode of emergence of the sporoplasm has given a clue to the probable arrangement of structures within the spore. All the evidence suggests that a polar capsule does not exist in these spores.
6. The spore wall of N. helminthorum has been clearly shown to consist of chitin, and to be of a single piece and not a bivalve structure.
7. Hymenolepis nana of mice and rats has been infected with this microsporidian by feeding H. nana-infected mice and rats with the spores. In these worms this organism shows all stages of development as seen in the natural host (Moniezia), but the spores assume a smaller size and the developmental cycle is probably of shorter duration. Even when heavily infected, the murine worms seem to be unaffected as far as movements and reaction to stimuli are concerned. In infected gravid segments the ova are found to be unattacked.
8. One specimen of Taenia saginata was experimentally infected with this microsporidian but here the duration of the infection was so short that early division stages only



were seen.

9. Two oribatid mites, Ceratoppia bipilis and Xenillus tegeocranus, were also infected with this organism, and in these the cells of the mid-gut and caeca were attacked. The spores in these were of a very much smaller size than the spores developing in H. nana.
10. The effects of the microsporidian on the cestode hosts have been discussed, and there is no evidence to show that this organism is pathogenic.
11. Nonema helminthorum has been reported from several countries, Germany, France, Gt. Britain, Yugoslavia, and Pakistan. A number of worms from a few other countries were also examined but no infected worms were detected. It is possible that as Moniezia and other anoplocephalid cestodes have a wide distribution, this microsporidian will eventually be reported from many other anoplocephalids from different countries.
12. The question of host-restriction of this microsporidian has been discussed. It is possible that if conditions for successful emergence of the sporoplasm are available in the intestines of the vertebrate hosts, then many tapeworms could be infected.

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PART II



**GIARDIA OVIS IN THE INTESTINE OF NEMATODIRUS  
FILLICOLLIS - A PARANEOXENOUS ASSOCIATION**

**A. S. DISSANAIKE**

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## **GIARDIA OVIS IN THE INTESTINE OF NEMATODIRUS FILLICOLLIS - A PARANEOXENOUS ASSOCIATION**

A. S. DISSANAIKE (\*)

During a survey of protozoa in helminths an examination of nematodes from sheep was made, and on several occasions specimens of *Nematodirus fillicollis* were found with their intestines teeming with flagellates belonging to the genus *Giardia*. As this was found to be quite a frequent phenomenon, a study of the behaviour and morphology of these forms was made, and a comparison of their structure with that of the *Giardia* inhabiting the small intestine of sheep was undertaken.

In the literature to date several reports are met with of flagellates encountered in the intestines of parasitic nematodes. THEILER and FARBER (1933, 1936), found *Trichomonas muris* of the laboratory white mouse in the intestines of *Aspicularis tetraptera* and *Syphacia obvelata*. In 1925 THOMSON described a *Giardia* «living and multiplying» in the intestine of the bursate nematode *Viannella* sp. from the small intestine of the viscacha (*Viscacia viscacia*). No cysts were seen in the intestines of these nematodes. Unfortunately in this case no specimen of *Giardia* either free or encysted was found in the faecal contents of the intestine of the viscacha, and although the morphological appearances of the *Giardia* suggested that it was identical with *G. viscaciae* described by LAVIER in 1923, no definite conclusions could be reached. The next report was by GRAHAM (1935) who found a *Giardia* in the intestines of several specimens of *Cooperia oncophora* obtained from a young bull. The infections were light and most numerous in the dilated portion of the intestine just anterior to the anus. Some of the specimens of *Giardia* were seen attached to the surface of the intestinal cells by their suckers. They were encountered in 6 out of 21 females and in none of 8 males. Here too no *Giardia* sp. was detected in the mucus of the bull's intestine, and so no comparison could be made in order to arrive at a definite conclusion regarding the nature of the association.

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(\*) *Department of Parasitology, London School of Hygiene and Tropical Medicine.*



## MATERIAL AND METHODS.

The nematodes were obtained from the London Metropolitan Cattle Market, Islington. They were brought with the intestinal contents, sorted out as quickly as possible and examined in saline. After the behaviour of the protozoa in the intestinal lumen of the worms was studied a number of worms were transferred into a tube of saline and left in the 37°C room in order to ascertain the length of survival of the worms and of the protozoa in them. Permanent stained preparations of the organism were made by dissecting the guts of the worms and making dry and wet smears. The former were stained in Giemsa stain for 12-16 hours and the latter were fixed, in Schaudinn's Fluid and stained in Heidenhain's iron-haematoxylin. A few infected worms were fixed in Carnoy's Fluid (6:3:1) sectioned at 5  $\mu$  and stained by the Giemsa-Colophonium method. Drawings and measurements were made from these preparations with the aid of a camera-lucida. *Giardias* obtained from the small intestine of sheep were stained with Giemsa stain after dry fixation as this was found to give the best results, especially when left in stain for over 12 hours. As will be described later, the motionless *Giardias* which were found in large quantities amongst the blood meal of several specimens of *Bunostomum trigonocephalum* were similarly prepared for study.

## OBSERVATIONS ON FRESH MATERIAL.

Since the first observation, specimens of *Nematodirus fillicollis* obtained from nearly every batch of sheep on different occasions showed *Giardia* sp. to a greater or less extent. It was found that although the worms could be kept alive in saline at 37°C for over 24 hours, the *Giardias* in them soon became motionless and were not recognisable after 4.5 hours. The table below (Table 1) summarises the findings on fresh material on different occasions.

TABLE I.

Date	No. worms examined	Males	Females	Time lapse since collection	Degree of Infect.
30. X.53	Over 20	nil	20 +ve	3 Hrs.	Heavy
11.XI.53	» 20	nil	20 +ve	1½ »	Heavy
18.XI.53	6	2 —ve	4 +ve	3 »	Light
26.XI.53	6	1 —ve	5 +ve	1½ »	Heavy
9.XII.53	20	3 —ve	17 —ve	3 »	nil
16.XII.53	1	nil	1 +ve	4 »	V. light

It is interesting to note that while nearly every *Nematodirus* examined contained *Giardia*, no males were seen to harbour it, although it must be admitted that the number of males examined was small. GRAHAM's findings in

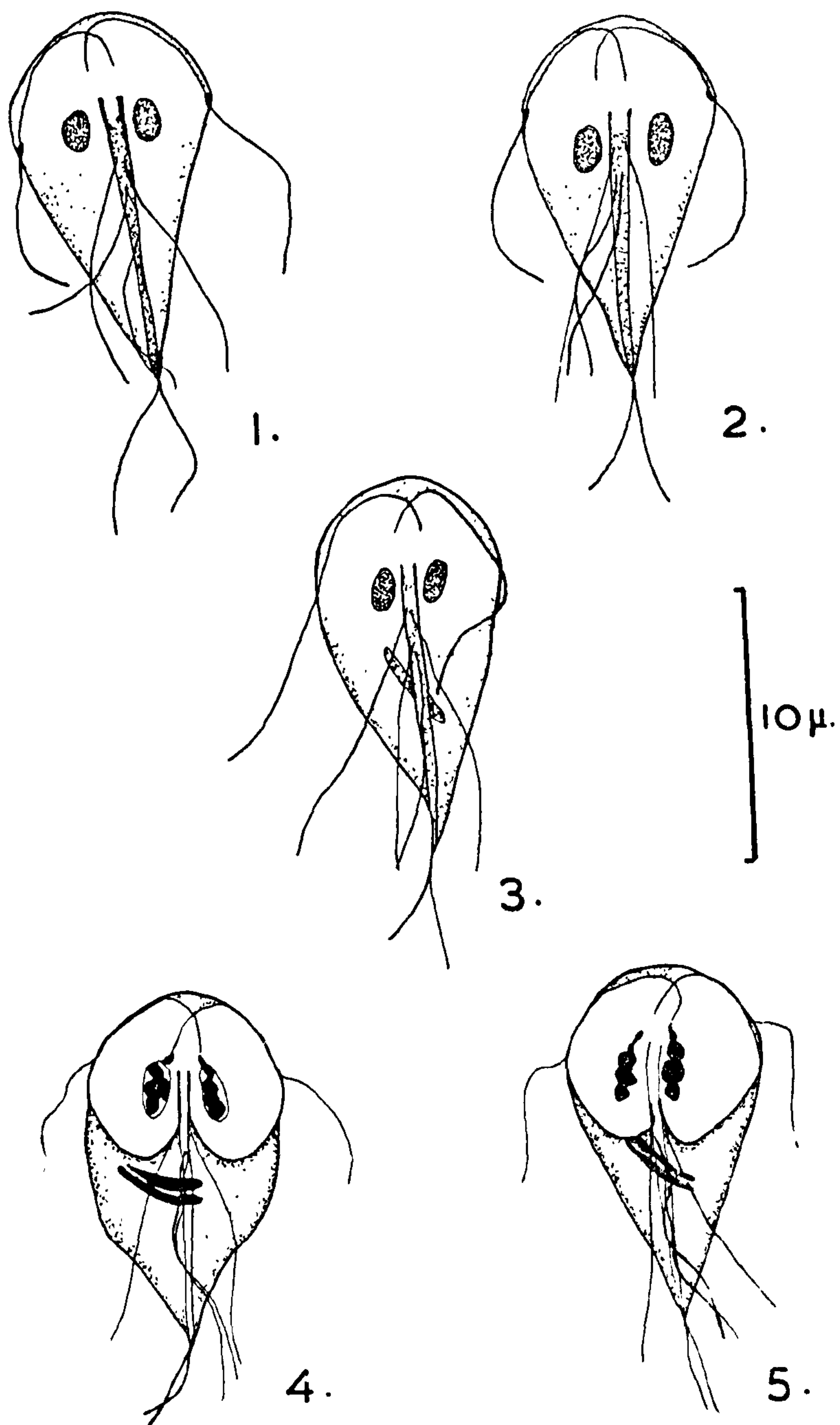


Fig. 1-3 Giardias from *Nematodirus fillicollis* (Giemsa).

Fig. 4-5 Giardias from *N. fillicollis* (Heidenhain).



the case of *Cooperia oncophora* were similar, for he found that none of eight males were infected. These observations may be explained as due to the physiological difference between the two sexes.

The difference in the degree of infection in worms examined on separate occasions, as seen in the table, may be explained as due to the time lapse between the collection of the worms and their examination. The absence of any infection in the batch of worms collected on 9.XII.53 may either mean that the worms were examined too late, or that the particular sheep from which these worms came was free from giardial infection.

Observations made on heavily infected worms showed that the *Giardias* were most numerous in the hind quarter of the intestine as was the case in GRAHAM's report. They were quite active either moving about in the intestinal lumen or attached to the surface of the intestinal cells by their ventral sucker-surface. In the latter case the rapid movements of the posterior flexible portions of the body of the organisms were very characteristic. No cysts were seen in any of the worms examined, nor was there any trace of the protozoa in any other part of the worms than the intestine.

#### DESCRIPTION OF THE GIARDIAS.

As the dry-fixed preparations stained in Giemsa stain were best for most details of structure, almost all the measurements and drawings were made from these. The *Giardia* sp. from *Nematodirus* (Figs. 1-5; 8-9) measured 13.2-14.25  $\mu$  in length by 6.9-7.6  $\mu$  in breadth. The ratio of length to breadth was 1.8-2.1. The averages of all these values were respectively 13.7  $\mu$ , 7.24 and 1.89.

The structure of the organism was quite typical. The antero-lateral pair of flagella arose from two separate granules from the contra-lateral sides a little anterior to the two nuclei, crossed at the anterior edge of the organism and remained attached to the lateral edge of the sucker till their point of emergence, where in a few specimens (Fig. 1-3) a thickened portion of the flagellum gave the impression of a secondary granule. This structure was first observed by BENSEN (1908) who called it the «basal granule», and he found these basal granules at the points of exit of the antero-lateral and caudal flagella. Since then KOROID and CHRISTIANSEN (1915), BOECK (1917, 1919) and SIMON (1921, 1922) have observed and remarked on these structures. Some of these workers consider them as secondary blepharoplasts, while others like HAIBA (1952) think they are produced by deposits of stain at the angle between flagellum and body. However, since these «basal granules» are so inconsistent, and when they do appear are seen to be definite thickenings of the flagella it is concluded that they are probably caused by the rapid lashing of the flagella just before death of the organism producing a denser spot at the junction of the attached and free portions of the flagella. The remaining

flagellar origins and arrangements did not show any deviation from the standard pattern. The axostyles were clearly double especially at the anterior ends where they began in a pair of blepharoplasts which were medial to those of the antero-lateral flagella.

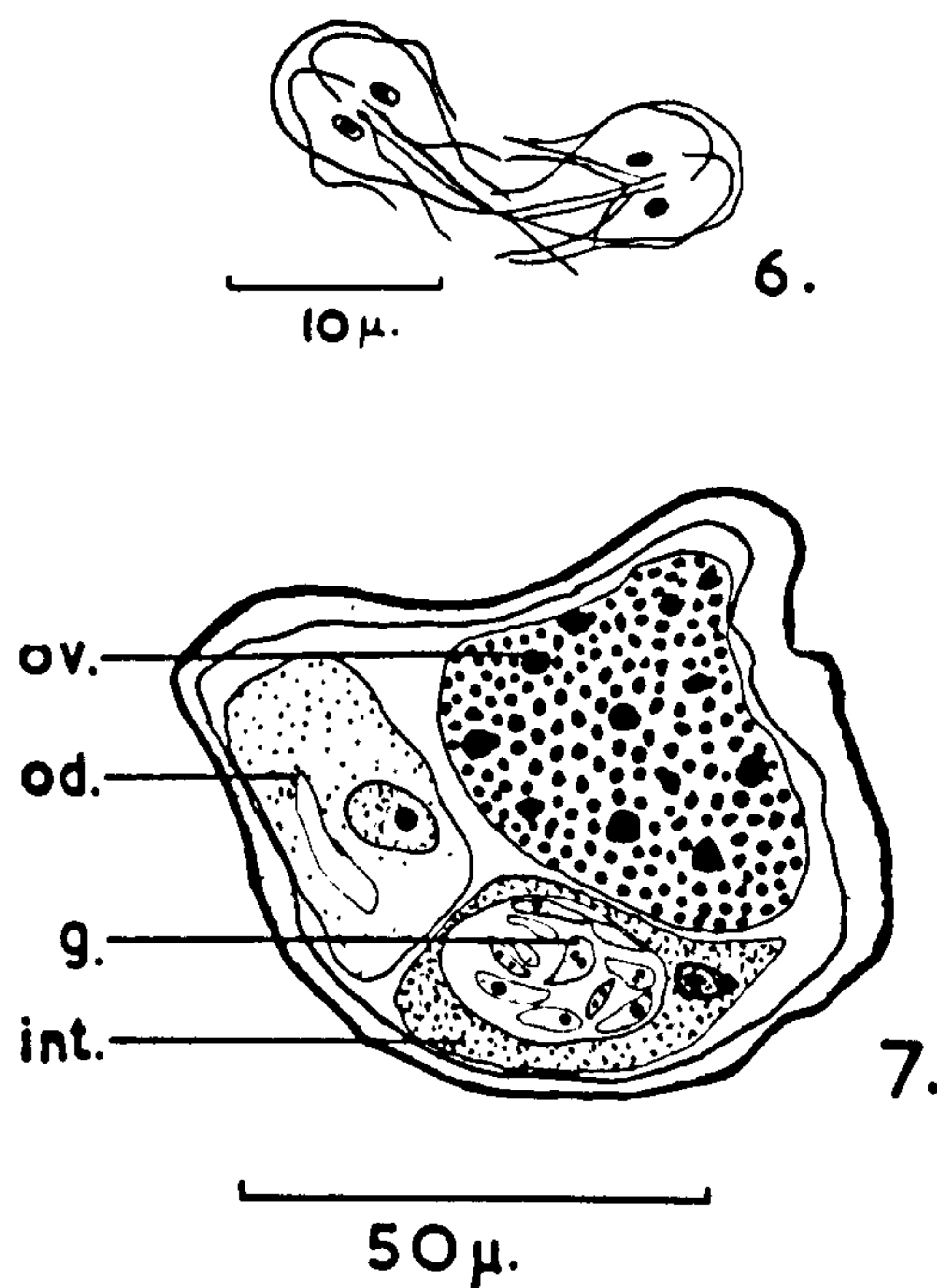


Fig. 6. Dividing form from intestine of *N. fillicollis* (Giemsa).

Fig. 7. Section of *N. fillicollis* showing Giardias in intestinal lumen (Giemsa-Colophonium).

Ov. - ovary.  
od. - oviduct.  
int. - intestine.  
g. - Giardias in lumen.

The nuclear details were not shown by Giemsa techniques appearing merely as homogeneous oval bodies measuring on an average 1.68 by 0.84  $\mu$ . In specimens stained in iron-haematoxylin they seemed to be made up of 3-4 compact chromatin masses. No rhizoplastic connections were found between nuclei and either of the blepharoplasts, although in Heidenhain stained smears a connection between the nuclei and the blepharoplasts of the antero-lateral flagella was sometimes seen. The sucking discs were only clearly seen in Heidenhain preparations, while the parabasal bodies which were mostly double, stained best in Heidenhain stained preparations and in Giemsa pre-



parations stained for 16 hours or more. They were placed at an oblique angle to the axostyles and measured on an average 1.9  $\mu$  in length.

In one smear preparation dividing trophozoite stages of the *Giardia* were seen (Fig. 6), which appeared to be the end stages of longitudinal binary fission.

Fig. 7 shows a section of an infected *Nematodirus* with the *Giardias* in the lumen of the intestine.

In 5 out of 14 specimens of *Bunostomum trigonoccephalum* large numbers of motionless *Giardias* were seen with the blood meal in the intestines of the worms. They were undoubtedly *Giardias* of sheep that had been ingested by the worms, and the fact that in *Bunostomum* they were not actively motile as was the case in *Nematodirus*, suggested that in *Bunostomum* at any rate they were *Giardias* of sheep that had been accidentally swallowed by the worms, and that the intestine of *Bunostomum* was not a suitable site for their growth and multiplication. It must also be mentioned here that several specimens of

TABLE II.

Giardia	Length	Breadth	Length: Breadth
<i>Nematodirus</i>	13.7(13.2-13.7) $\mu$	7.2(6.9-7.6) $\mu$	1.89(1.80-2.05)
<i>Bunostomum</i>	14.39(13.05-15.5) $\mu$	7.82(7.5-8.0) $\mu$	1.85(1.75-1.95)
Sheep (Present paper)	13.1(12.4-13.8) $\mu$	7.08(6.9-7.35) $\mu$	1.85(1.79-1.97)
Goats (NIESCHULZ, 1924)	14.0(11.0-17.0) $\mu$	7.5(6.0-8.5) $\mu$	1.8(1.82-2.0)

*Trichostrongylus* spp. from sheep were examined but no *Giardias* were seen in them. Table II gives a comparison of the measurements of *Giardias* from *Nematodirus*, *Bunostomum* and from sheep. The rather higher values for those of *Bunostomum* may be due to the fact that the *Giardias* were already dead for some time and hence spread out more on fixation. Apart from this they all seem the same. Figs. 8-13 show *Giardias* from *Nematodirus*, *Bunostomum* and sheep drawn to the same scale for comparison.

#### DISCUSSION.

There are two questions to be settled before deciding on the true significance of the above findings. First, whether the *Giardia* found in the intestine of *Nematodirus fillicollis* is a new species peculiar to it, or, secondly, if it is in fact the *Giardia* of sheep that has been accidentally swallowed and has found the intestine of the nematode a suitable site to live in.

The two previous records of *Giardia* occurring in the intestines of the trichostrongyles *Viannella* sp. (THOMSON 1925), and *Cooperia oncophora* (GRAHAM 1935), have already been mentioned. In these cases the writers were

unable to decide definitely whether they were dealing with *G. viscaciae* Lavier, 1923 and *G. bovis* Fantham, 1921 respectively. THEILER and FABER (1938 and 1936) were certain that the trichomonads they found in the intestines of *Aspiculuris tetraptera* and *Syphacia obvelata* were the same as *T. muris* of

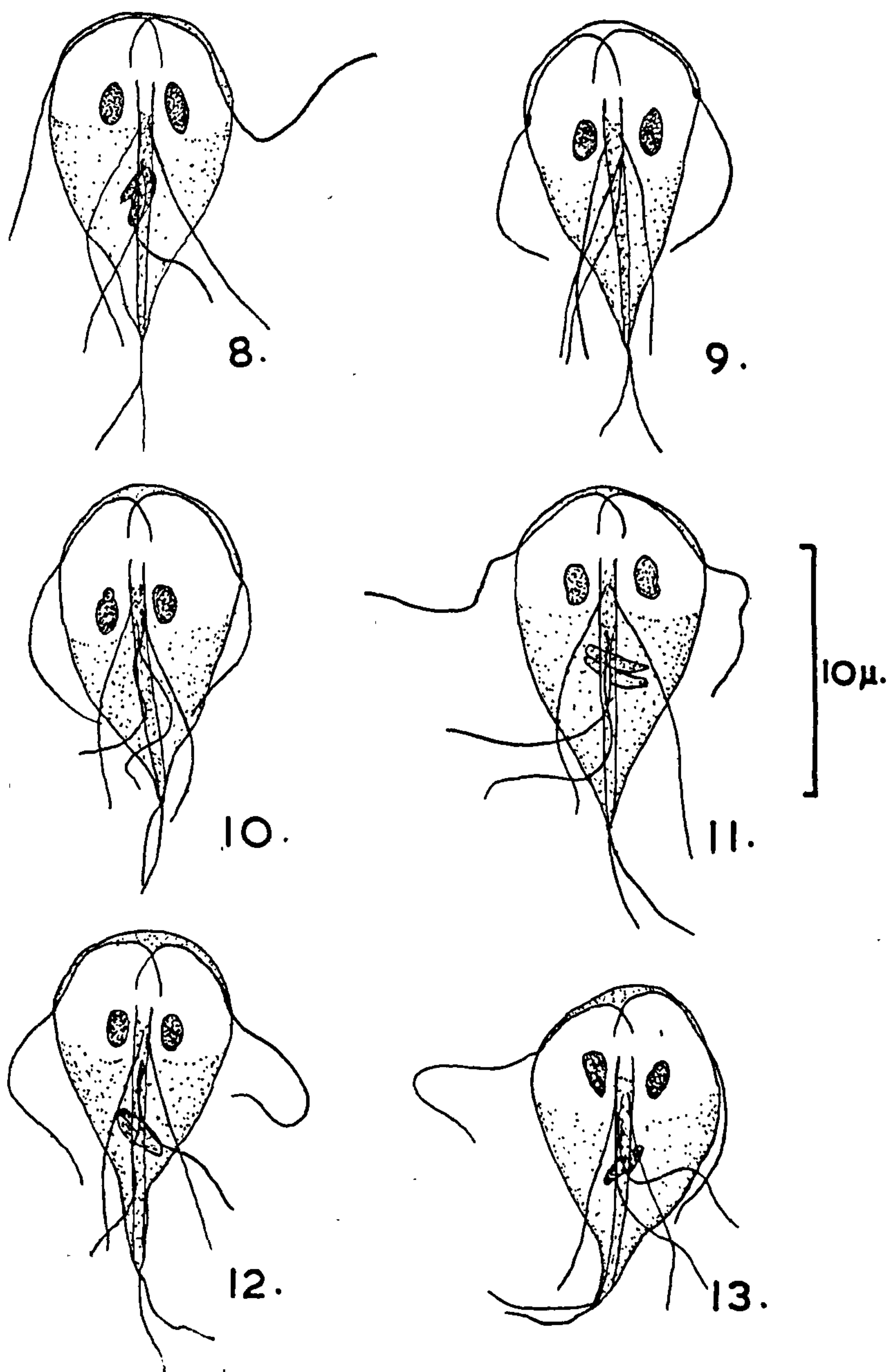


Fig. 8-9. Giardias from. *N. fillicollis*.  
 Fig. 10-11. » » *B. trigonocephalum*.  
 Fig. 12-13. » » *sheep*.



the mouse. CALLOT (1942) described a case of *Giardia muris* in the intestinal caeca of the trematode *Echinostoma revolutum* from the intestine of the white mouse. He refers to this association as one of *paraneoxenie* a term originally proposed by BRUMPT and LAVIER (1936) for the type of hyperparasitism exhibited by an opaline, *Zelleriella* sp. in the body of which an amoeba was found, both the opaline and the amoeba being normally inhabitants of the digestive tract of a toad *Paludicola signifera*. This term was used to describe the type of association where one intestinal parasite invades the digestive tract of another intestinal parasite, that shares the same habitat in the host's intestine, thereby making this parasite its new host. CALLOT was of opinion that since *Giardia* is normally very difficult to culture, the intestinal caeca of the trematode must have been a suitable site for it to live in and even multiply. It is therefore reasonable to assume that in this particular case too we are dealing with an example of a paraneoxenous association. The morphological appearances of *Giardia* in the intestines of *Nematodirus* and of sheep are undoubtedly the same, as the various figures and table indicate. Furthermore if *Giardia* in *Nematodirus* was peculiar to it one would have expected to find cysts in the intestines of the worms.

Finally it is necessary to clarify the nomenclature of *Giardia* of sheep. This organism was first observed by GRASSI (1881) who mentioned its existence in sheep but did not give it a special name or description as he probably considered it as identical with the human species which he called *Megastoma entericum*. It was again stated as occurring in sheep by HEGNER (1924) who also did not name or describe it. The only description met with in the literature was by TURNER and MURNANE (1932) who reported it from sheep in Victoria, Australia, gave a description of the organism, and compared its measurements with those of some other mammals. They concluded that «the form described by us resembles the other species from mammals, but is smaller than the human form which GRASSI apparently regarded it as identical. It is about the same length, but is not so broad as *G. caprae* described by NIESCHULZ (1923) in the goat, and most closely approximates the *G. canis* of the dog». They did not name the organism, but NEVEU-LEMAIRE (1943) called it *G. ovis* without giving any description. The measurements of the *Giardia* sp. described in the present work fall within the ranges given by TURNER and MURNANE, and although they agree very closely with the measurements of *G. caprae*, ANSARI'S (1952) suggestion that it is probably synonymous with *G. caprae* cannot be accepted until by cross-infection experiments it can be clearly shown that the two species are identical. For the present then *G. ovis* NEVEU-LEMAIRE, 1943 must be regarded as valid.

It can therefore be concluded that present report of a *Giardia* in *Nematodirus fillicollis* of sheep is yet another example of a paraneoxenous association where the *Giardia* is none other than *G. ovis* of sheep.

## ACKNOWLEDGMENTS.

I wish to express my thanks to Professor P. C. C. GARNHAM, Professor J. J. C. BUCKLEY, and Dr. P. L. LE ROUX for their help and advice in this work. To, Mr. E. F. McCLEARY, Chief Veterinary Officer, Mr. R. G. HALCROW, Assistant Veterinary Officer, and Mr. H. T. YELLAND, Meat Inspector, of the London Metropolitan Cattle Market, Islington for their kindness in providing the facilities for obtaining the material from sheep.

## RIASSUNTO

L'Autore descrive una *Giardia* che vive e si moltiplica nell'intestino del trichostrongilo della pecora, *Nematodirus filicollis*. Paragonando questo parassita con *Giardia ovis*, ottenuto dall'intestino della pecora, viene concluso che le due forme sono identiche.

L'Autore considera questo come un caso di associazione paraneoxenica, di cui si hanno altri esempi nella letteratura.

## SUMMARY

A *Giardia* living and multiplying in the intestine of the sheep trichostrongyle *Nematodirus filicollis* is described. A comparison of this parasite with *Giardia ovis* obtained from the intestines of sheep has been made and it is concluded that the two forms are identical.

Further examples of this type in the literature have been reviewed, and this is regarded as yet another instance of a paraneoxenous association.

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## PART III

\* A New Schizogregarine Triboliocystis garnhami n.g., n.sp.  
and a New Microsporidian Nosema buckleyi n.sp., from  
the fat body of the Flour Beetle Tribolium castaneum .

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( \*<sub>m</sub> Journal of Protozoology, In press )

The larvae of a culture of Tribolium castaneum sent by Mr. J.M.Holborn of the Cooper Technical Bureau, Berkhamsted , to Professor P.C.C.Garnham in November 1954, were found to be heavily parasitized by a schizogregarine. Professor Garnham kindly handed over the material to me for further study. The parasite proved to belong to a new genus ~~Triboliocystis~~ I propose the name Triboliocystis garnhami n.g., n.sp. , in honour of Professor Garnham. A microsporidian found in a single pupa from this culture was considered to belong to a new species , and I propose to call it Nosema buckleyi n.sp., after Professor J.J.C.Buckley.

Several parasitic protozoa have so far been reported from various species of Tribolium . White (1923) reported a neosporidian from Tribolium confusum and T. ferrugineum but gave no description nor any diagrams of the parasite and its nature remains obscure. Riley and Krogh (1927) found a coccidian from the same two hosts and said that it closely resembled Adelina mesnili Perez,



1899. What was probably the same organism was found in the fat body of T. ferrugineum by Ehatia ( 1937 ) who gave a full description and called it Adelina tribolii . Recently Weiser ( 1953 ) described two new parasites from T. castaneum and T. confusum . One of these , a schizogregarine, he described in detail and named Farinocystis tribolii . The other was a microsporidian which he believed to be a Nosema , and called Nosema whitei .

### Material and Methods

Studies on fresh material were carried out by crushing the larvae and other stages in normal saline. The oocysts or spores were measured in such preparations. Most of the stages of the schizogregarine were studied from smears and sections fixed in Duboscq-Brasil fixative and stained in Heidenhain's iron-haematoxylin or Giemsa stain . Sections were cut at 5-7  $\mu$ . thickness after double-embedding in celloidin and paraffin wax using Peterfi's method. All drawings were made with the aid of a camera-lucida.

### Description of the Schizogregarine

The description of this parasite is given under the following headings - schizogony, gametogony and sporogony . As with most of the schizogregarines described

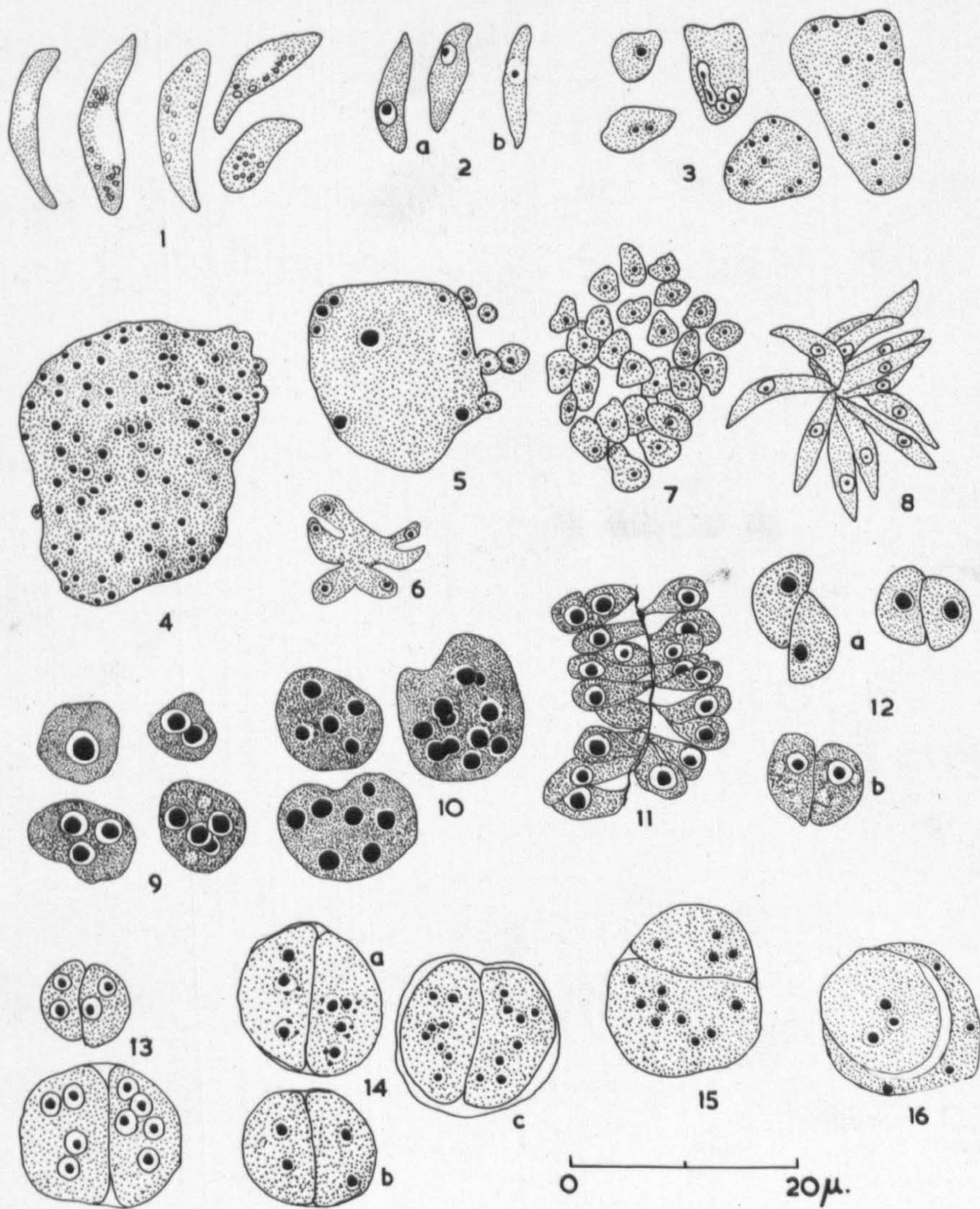
Explanation of Plate 1.

1. Trophozoites in saline preparation.
2. " in sections (a) Giemsa (b) Heidenhain.
- 3-5. Micronuclear schizonts.
6. Micronuclear schizont with finger-like projections.
- 7,8. Merozoites resulting from micronuclear schizogony, elongating later to form trophozoites. (S)
9. Macronuclear schizonts ( Giemsa)
10. " " ( Heidenhain)
11. Merozoites formed by macronuclear schizogony.
12. Gametocytes uninuclear at association (a) Heid: (b)Giem
13. Gametocytes with nuclei divided ( Giemsa)
14. " ( Heid:) a. showing extra chromatin bodies  
b. binuclear stage  
c. 8-nuclear stage.
15. Gametocytes showing 8 nuclei in one.
16. Associated gametocytes with one in a cup-shaped depression formed by the other.

( Fig. 2-16 drawn from sections.)



# PLATE 1





so far , two types of schizonts are seen , a micronuclear and a macronuclear.

### 1. Schizogony

The trophozoites ( Fig. 1-2 ) are vermicular and spindle -shaped or fusiform measuring in fresh preparations 13.2 - 19.7  $\mu$ . by 2.3 - 3.4  $\mu$ ., and having a broader and blunter posterior end. A few large refractile granules are present in the cytoplasm of some of the fresh preparations. of trophozoites. In stained preparations ( Fig. 2) the nuclei of these trophozoites are nearer the broad end and contain a large central karyosome. These trophozoites soon become round and the nucleus in each divides. The micronuclear schizonts thus formed, measured in sections, range from 7-25  $\mu$  in diameter and the largest of these (Fig. 4) has over a hundred nuclei. Figures 3-6, and Fig. B show various appearances of micronuclear schizonts. In a few of these schizonts (Fig. 5) the nuclei are not all of the same size and some of the larger nuclei , approaching the size of the nuclei of the macronuclear type, could possibly be the precursors of the macronuclear type of schizont. The merozoites that are produced by this schizogony are more or less globular or ovoidal at first, and



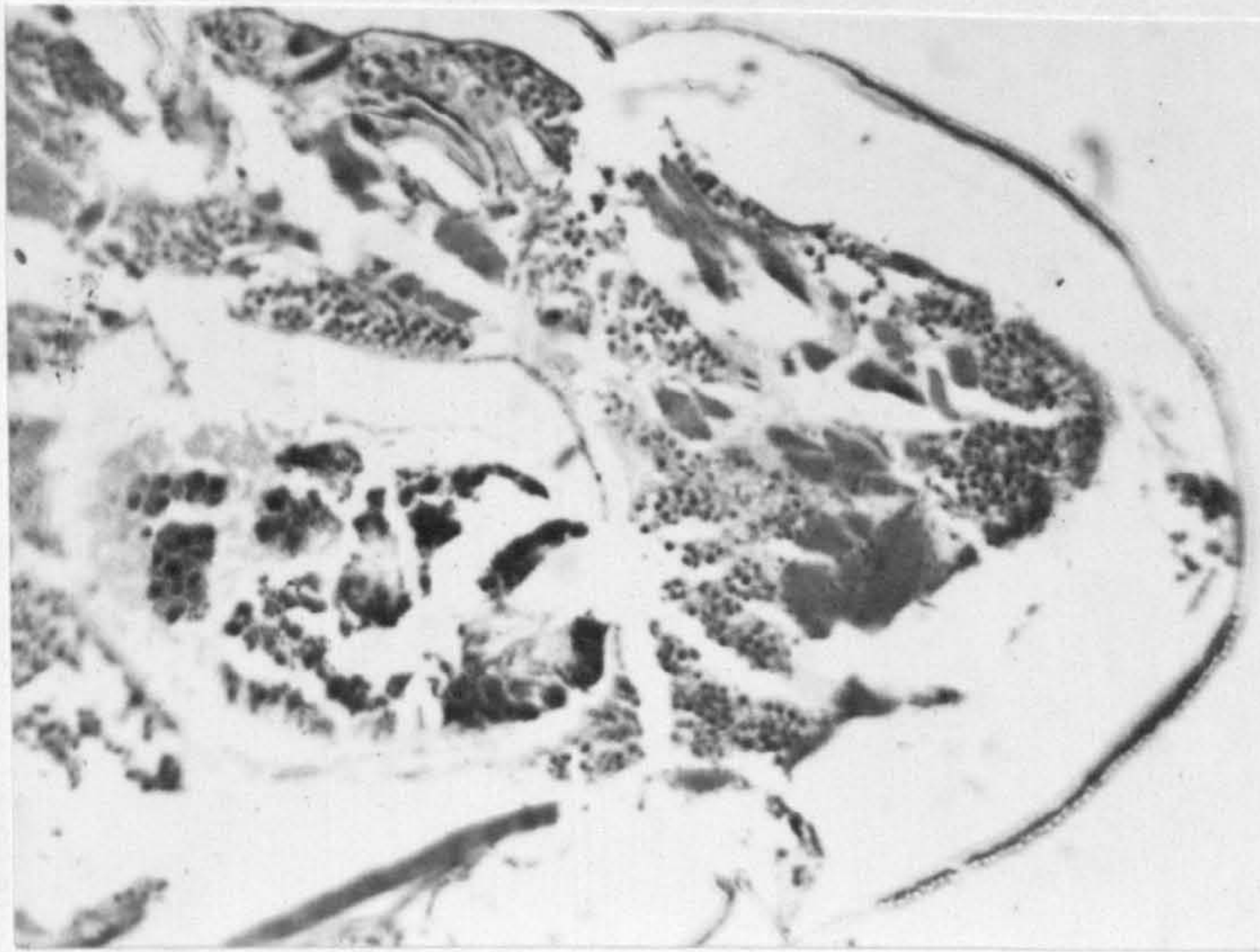


Fig. A.  
 Section of an infected larva of T. castaneum  
 showing infiltration and destruction of muscle.  
 Giemsa-Colophonium ( X 100 )

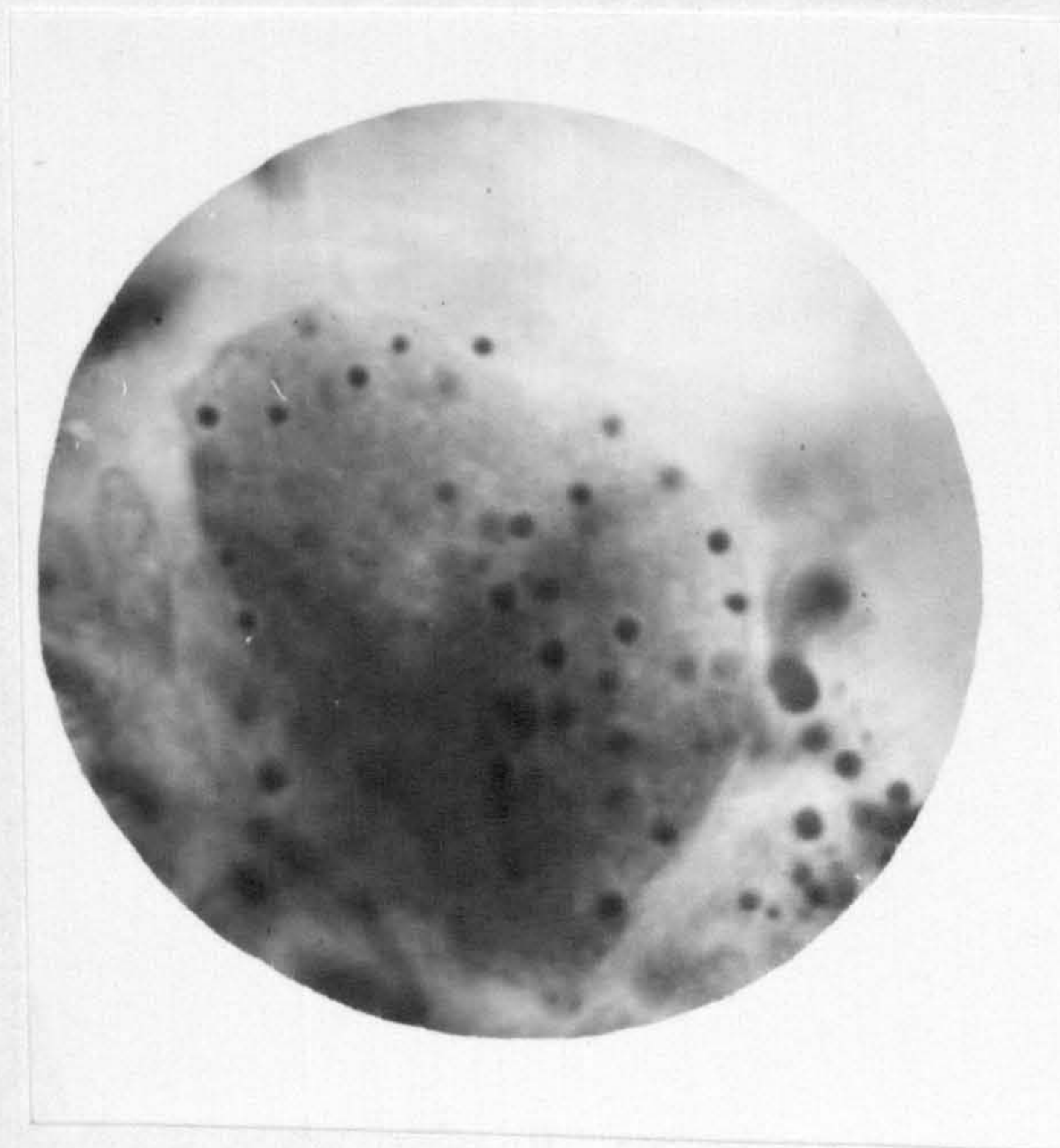


Fig. B.  
 A micronuclear schizont from a section of fat-body  
 of an infected larva ( Heidenhain ; X 2,000 )



some are budded off from finger-like projections at the periphery of the schizont ( Fig. 6) . The majority are, however, formed by the rupture of a multinucleate mass ( Fig. 7). As many as twenty-six merozoites were seen formed from one such schizont in this manner. These merozoites soon elongate and become trophozoites once again ( Fig. 8 ).

The macronuclear schizonts ( Fig. 9-10 and Fig. C. ) which are probably derived from the larger nuclei of the micronuclear type, have deeply staining cytoplasm and their nuclei are about twice the size of those of the micronuclear type, measuring 2  $\mu$ . in diameter. The smallest of these macronuclear schizonts is about 5  $\mu$ . in diameter and somewhat globular. The larger ones are of irregular outline and the largest seen was 16  $\mu$ . by 12  $\mu$ . There are never as many nuclei as in the micronuclear schizonts, the maximum number observed being twelve. The nuclei of these macronuclear schizonts move to the periphery where the merozoites are budded off to give rise to the gametocytes (Fig.11).

#### Gametogony

Gametocytes are uninuclear when they associate in pairs ( Fig. 12 and Fig. D ) ; a little later their



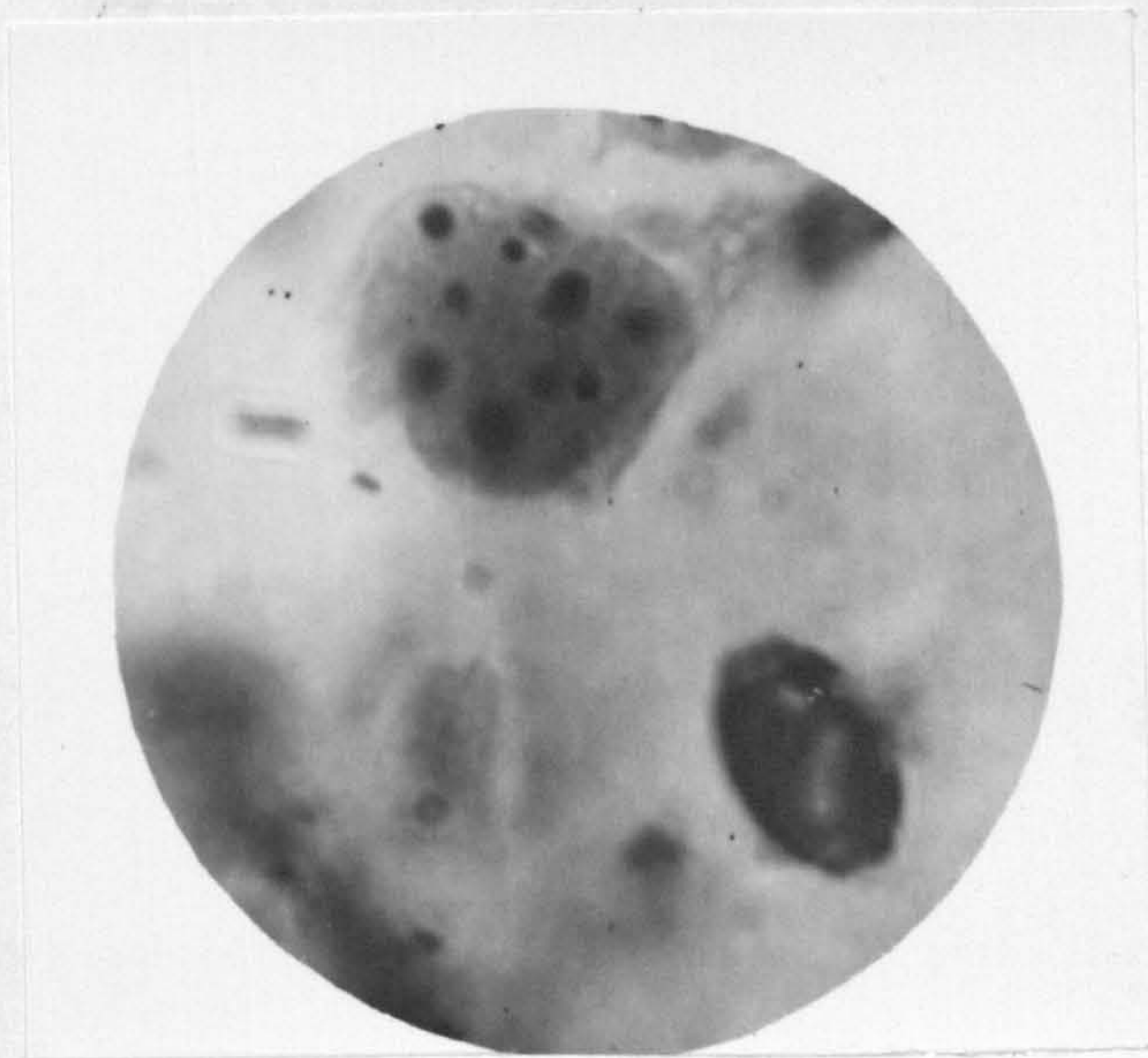


Fig. C.  
Macronuclear schizont from a section of fat-body  
of a larva ( Heidenhain ; X 2000. )

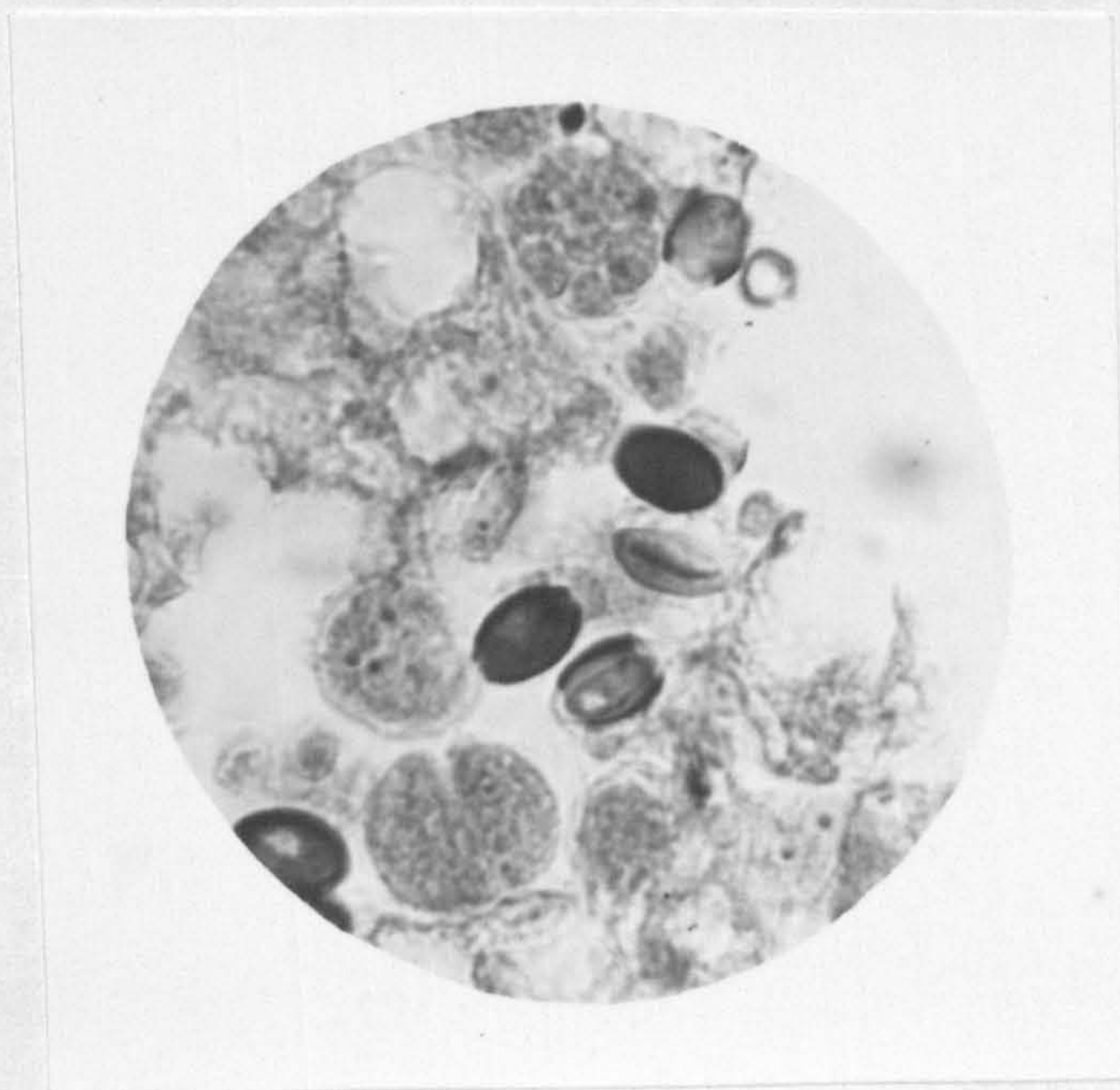


Fig. D.  
Section of fat-body of infected larva showing a  
pair of uninuclear gametocytes in association.  
Heidenhain ; ( X 900. ).



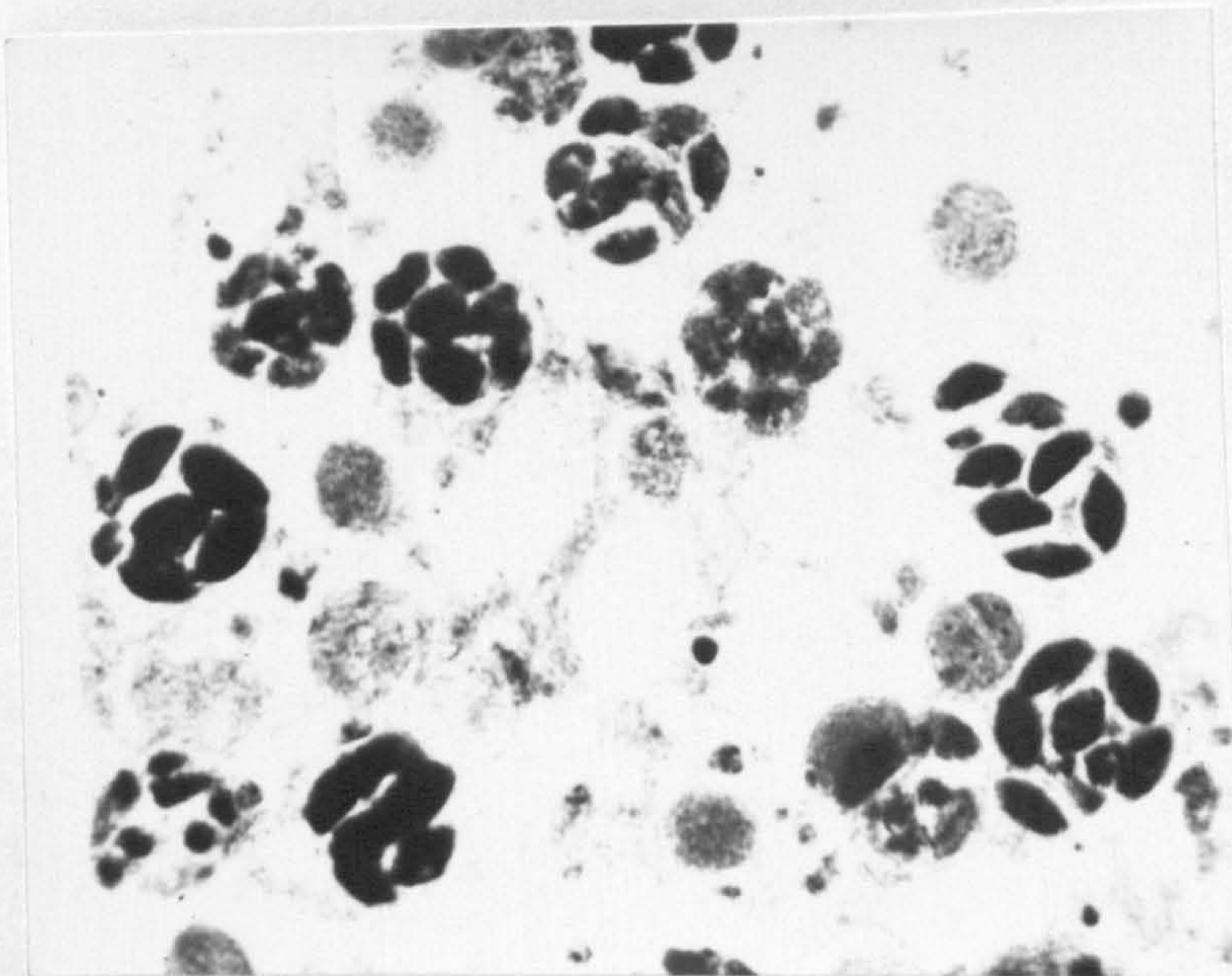


Fig. E.  
Smear preparation of infected fat-body showing  
two gametocyte pairs in association and several  
gametocysts containing oöcysts. (Heid: X 600 )



Fig. F.  
Oöcysts in section showing one in which some of the  
sporozoites have escaped , and one just about to emerge.  
( Giemsa-Colophonium X 2000 )

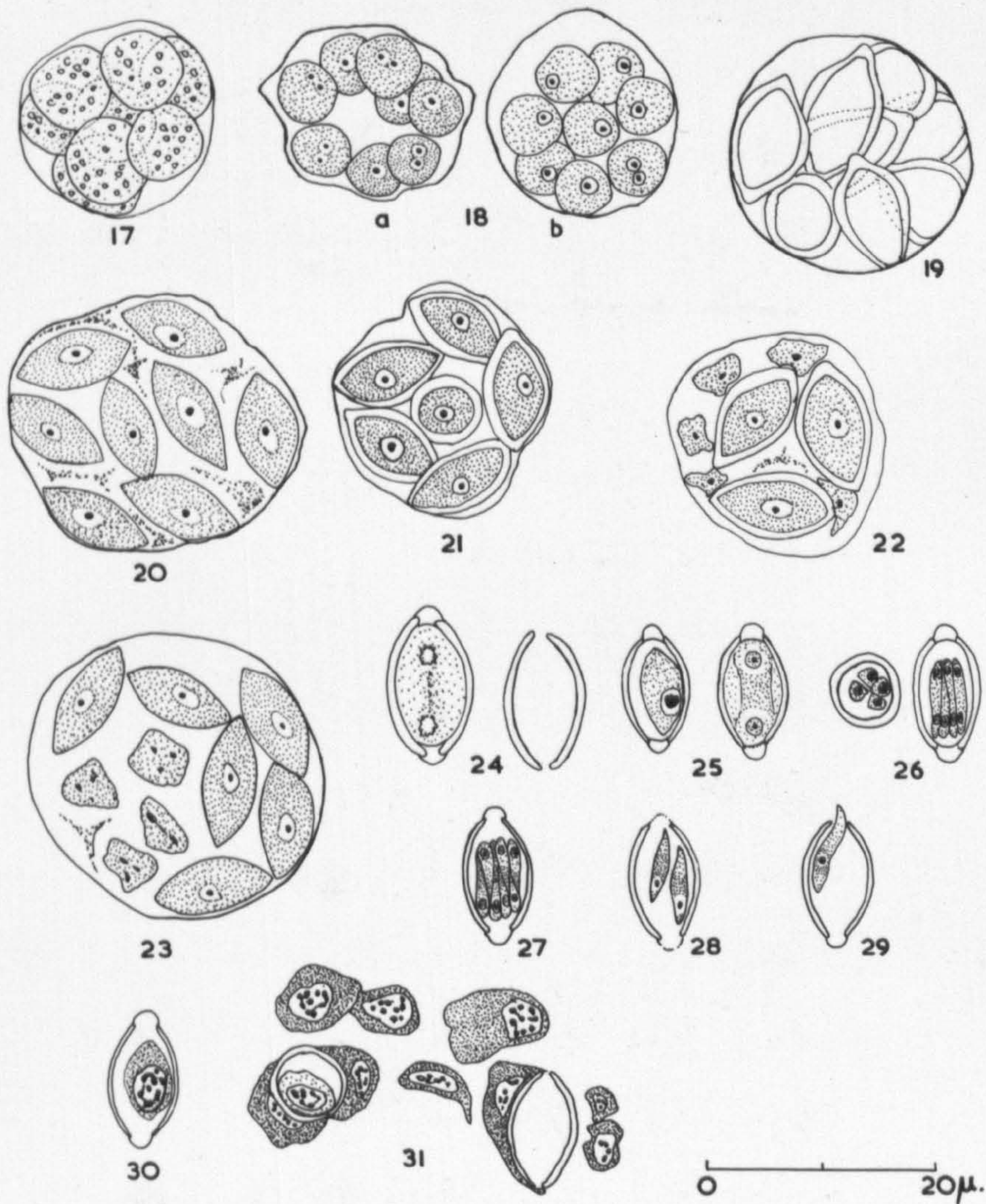


Explanation of Plate 2

17. Gametocyst from saline preparation with 8 zygotes.
18. Gametocysts in section (a) Heid: (b) Giemsa, with 8 zygotes in each . Some nuclei still unfused.
19. Gametocyst in saline showing 8 oöcysts.
20. Smear stained in Heidenhain showing gametocyst with 8 oöcysts.
21. Gametocyst in section showing oöcysts (uninuclear)
22. " from a smear (Heid:) showing only 3 oöcysts continuing to develop.
23. Same showing 6 oöcysts developing.
24. Two oöcysts in saline, one empty with polar caps detached.
25. Uni- and bi-nuclear stages of oöcysts from section (Giemsa).
26. T.S. and L.S. of oöcysts at 4-cell stage. from section (Giemsa)
27. Oöcyst with 8 sporozoites. (Section , Giemsa)
28. " with two sporozoites remaining(" )
29. " with sporozoite emerging . ( " )
30. Empty oöcyst occupied by a host phagocyte.
31. Host phagocytes and oöcysts engulfed by them.



PLATE 2.





nuclei divide three times to give rise, as a rule, to eight nuclei . Occasionally gametocytes are observed with nine or ten nuclei in each ( Fig. 15 ). This is clearly due to one or two of the daughter nuclei undergoing an extra division , and explains the occasional presence of more than the normal number (eight) in a gametocyst. An interesting feature of these gametocyte nuclei is that after the first nuclear division, in addition to the karyosome, two smaller chromatin bodies can be made out (Fig. 14 a ). This appearance of the nuclei is best observed in Heidenhain-stained sections, and is not found in any of the other stages except in a few macronuclear schizonts where one or two of these extra chromatin bodies are noticed.

When eight nuclei have been formed the cytoplasm of the two associates fuse at the line of contact and it is only at about this time that the gametocyst wall is secreted. Although as a rule the gametocyte pairs lie side by side, each appearing somewhat hemispherical, a number are seen in which one associate remains spherical and lodged within a cup-shaped depression of the other ( Fig. 16 ).

Gametes are apparently formed by a process

of internal budding ; fertilization is probably by fusion of gametes of opposite gametocytes, which is followed by fusion of their nuclei( Fig. 18 ). In this way eight zygotes are formed in each gametocyst ( Fig. 17 -18 ) and around each zygote a resistant coat is secreted producing a fusiform oöcyst .

### 3. Sporogony

The formation of eight oöcysts within each gametocyst can be considered the normal occurrence, but a large number of gametocysts are seen, especially in smears, with 3, 4, 5, 6, 7, 9, and 10 oöcysts in them. The probable explanation of this is given later ( Fig. 22-23 ) .

Oöcysts are typically navicular (Figs 24-29 and Fig. F. ) with the two poles rounded and capped with a less resistant membranous material. They measure in the fresh 13.3 - 14.3  $\mu$ . by 6.6 - 7.7  $\mu$ . Actual division of the zygote nucleus was not seen , but in some fresh smears and in sections, oöcysts with two nuclei at opposite poles were found (Fig. 24- 25). It appears that the first division brings the nuclei to opposite poles and that the next two divisions occur with the resulting nuclei remaining at the poles. The second and third divisions are at right angles to each other . Thus eight nuclei are formed in all,



and now two cytoplasmic cleavages occur longitudinally giving rise to four elongate cells each with two nuclei at opposite poles. The next cleavage is oblique and results in the formation of eight sporozoites as shown in Figure 27. Each sporozoite has a broader end containing the nucleus and a narrower tapering end. Sporozoites in sections measure 6 - 7.8  $\mu$ . by 1.2 - 1.4  $\mu$ .

In some heavily infected larvae several empty oöcysts were found, which suggests that oöcysts hatch in the same host. In Mattesia dispora Neville (1930) noted that autoinfection in this manner took place, and Misgrave and Mackinnon (1933) and Finlayson (1950) confirmed his observations. Weiser (1954) however, disagrees with this interpretation. I have seen several oöcysts with only two or three sporozoites remaining in them, and a few with one of these sporozoites escaping from the oöcyst (Fig. 29 and Fig. F.). Another observation that supports the view that oöcysts hatch in the same host (autoinfection) is that phagocytes are often found inside empty oöcysts. This suggests that the sporozoites have emerged and that phagocytes have entered through their place of exit.

### Effects on the Host

All the larvae examined were found to be infected to a greater or lesser extent. Pupae were rarely found, but two contained oöcysts ; one of these had, in addition to the oöcysts , the spores of the microsporidian to be described later. Although a large number of adults were examined by crushing and by sectioning, only one - a dead one - was found to contain oöcysts. It appears from these observations that the infection reaches its height in the late larval instars where it is fatal. A few that are not so heavily infected progress to the pupal and even to the adult stage , and of these some eventually die .

In infected larvae the fat body is the primary site of infection, but in some heavily infected specimens various stages of the parasite are seen in the haemocoel and also in the muscle, where infiltration and destruction of tissue are seen ( Fig. A ). The nervous system, gut and malpighian tubules are never affected.

As mentioned earlier host phagocytic cells are present in large numbers in those infected larvae that show empty oöcysts. Some of these phagocytes engulf the empty oöcysts ( Fig. 31 ) while others are seen inside



them ( Fig. 30 ).

### Discussion

Before considering the systematic position of Triboliocystis garnhami it is necessary to review briefly the classifications that have been put forward from time to time for the schizogregarines. Léger and Duboscq ( 1908 ) divided schizogregarines into two groups, the Monosporea and the Polysporea according to the number of oöcysts present in each gametocyst. In the same year Fantham (1908) on purely ecological grounds, divided them into Ectoschiza ( with extracellular schizogony ) and Endoschiza ( with intracellular schizogony ). Keilin (1923) regarded the schizogregarines as a composite group and believed that the different genera were evolved from different families of the Eugregarina by independently acquiring a schizogonic cycle. According to Naville ( 1930 ) the fundamental basis of classification should be the chromosome cycle . In monocystid types he said that reduction occurs immediately preceding gamete formation, and so they are diploid, while in the polycystid forms reduction is post-zygotic and so they are haploid. Unfortunately the chromosome cycle has not been worked out in all schizogregarines and such a classification is not practicable at present. Grassé (1953) suggested an

• Since this paper has gone to press, Weiser has published a correction to his new classification. In accordance with the Rules of Nomenclature he has renamed the two families Ophryocystidae and Caulleryellidae ( in place of Dischizae and Monoschizae. ).



entirely new classification for the schizogregarines . He divided the Gregarinomorpha into three groups -

- i. Archigregarina
- ii. Eugregarina
- iii. Neogregarina

In the Archigregarina he placed what he believed to be the more archaic schizogregarines with true schizogonies; while those schizogregarines with an accessory schizogony he called Neogregarina . It is questionable whether such a separation of the schizogregarines is justifiable .

Recently Weiser ( 1954 a, 1955 ) has suggested a classification based on a study of their developmental cycles, which seems a natural one. He divides them into two main families, the Dischizae and the Monoschizae<sup>\*</sup>, to separate the two main groups one with two schizogonic cycles and the other with only one. The Dischizae, to which the present parasite belongs, will now be discussed. Weiser divided the Dischizae into two tribes or sub-families , the Ophryocystinae and the Machadoellinae . The main difference between them is that in the former the products of the second schizogony are morphologically distinguishable from the trophozoites, the gametocytes are uninuclear at the beginning of association and the oöcysts are fusiform with thickened

poles . In the Machadoellinae gametocytes are multi-nuclear by the time they associate and the products of the second schizogony resemble trophozoites. Tribollocystis clearly belongs to the sub-family Ophryocystinae. The separation into genera in this sub-family rests mainly on the number of oöcysts within each gametocyst, and according to the number of times the nuclei of the gametocytes divide, several possibilities exist. Hence genera with 1, 2, 4, 8, 16, 32, 64, 128, and so on are to be expected. These numbers are the normal numbers for each genus . But abnormalities of division of nuclei of gametocytes such as suppression of one division in one or all the nuclei of a gamont, or division of a daughter nucleus beyond the limit for that genus, can eventually produce a smaller or larger number of oöcysts than the normal. This would explain the varying numbers of oöcysts met with in gametocysts in the present species. When the number of oöcysts is less than the normal ( Fig. 22-23 ) another explanation is possible. In such gametocysts, although the normal number of eight zygotes is formed, only a few continue to develop while the rest degenerate. This is apparently due to pressure, and so in heavy infections it is likely that the average number may be less than eight ( Fig. E ).



These abnormalities in the numbers of oocysts have been noted by many workers who have given different explanations for the phenomenon. For instance Leger (1907) explained the occurrence of two 'spores' in species of Ophryocystis, where the normal number is one, as due to the parthenogenetic development of each of the two gametes formed. Weiser (1954) synonymised Mattesia dispora Naville, 1930 and Coelogregarina ephestiae Ghelelovitch, 1947, the former with two oöcysts and the latter with one, by explaining that the former type occurs in old chronic infections and the latter in fresh infections.

One member of the Monoschizae too possesses eight oöcysts in a gametocyst. This genus Caulleryella Keilin, 1914 however, cannot be confused with Triboliocystis for many reasons. Apart from the fact that Caulleryella has only one type of schizont, its oöcysts are rounded with the sporozoites attached to one pole, and the trophozoites are quite different.

Finally, my observations suggest that autoinfection takes place in Triboliocystis garnhami confirming those of Naville, Musgrave and Mackinnon, and Finlayson.

#### Description of the Microsporidian

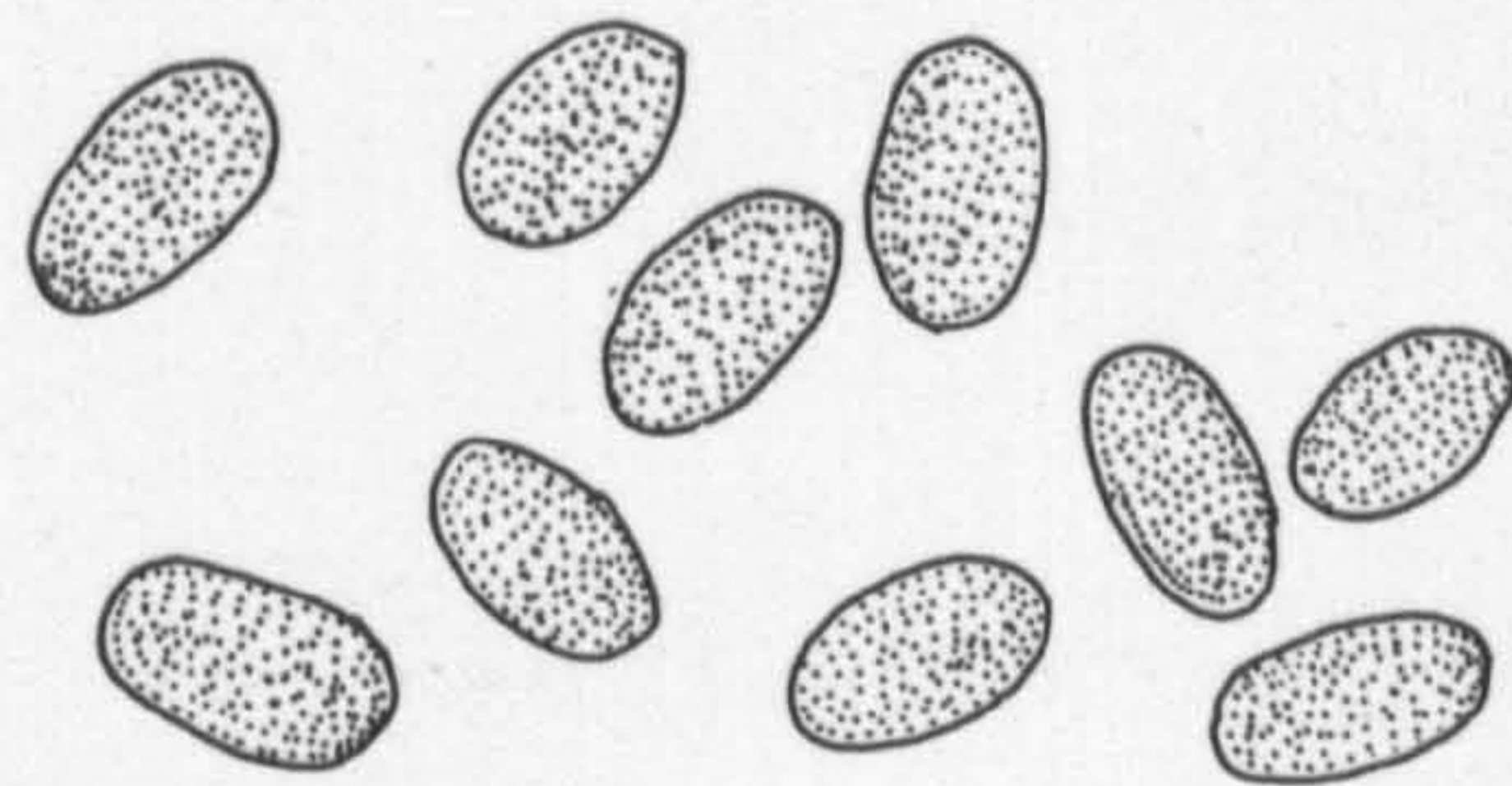
One pupa in the present material showed in

Explanation of Plate 3

- 32. Fresh spores of N. buckleyi.
- 33. A spore with extruded filament showing sporoplasm at the tip.
- 34. Two spores with extruded filaments stained in Giemsa stain.

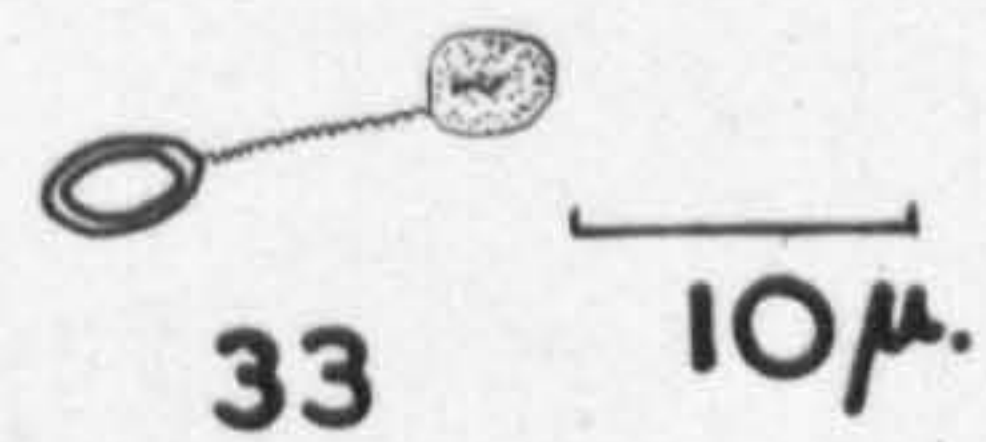


PLATE 3.



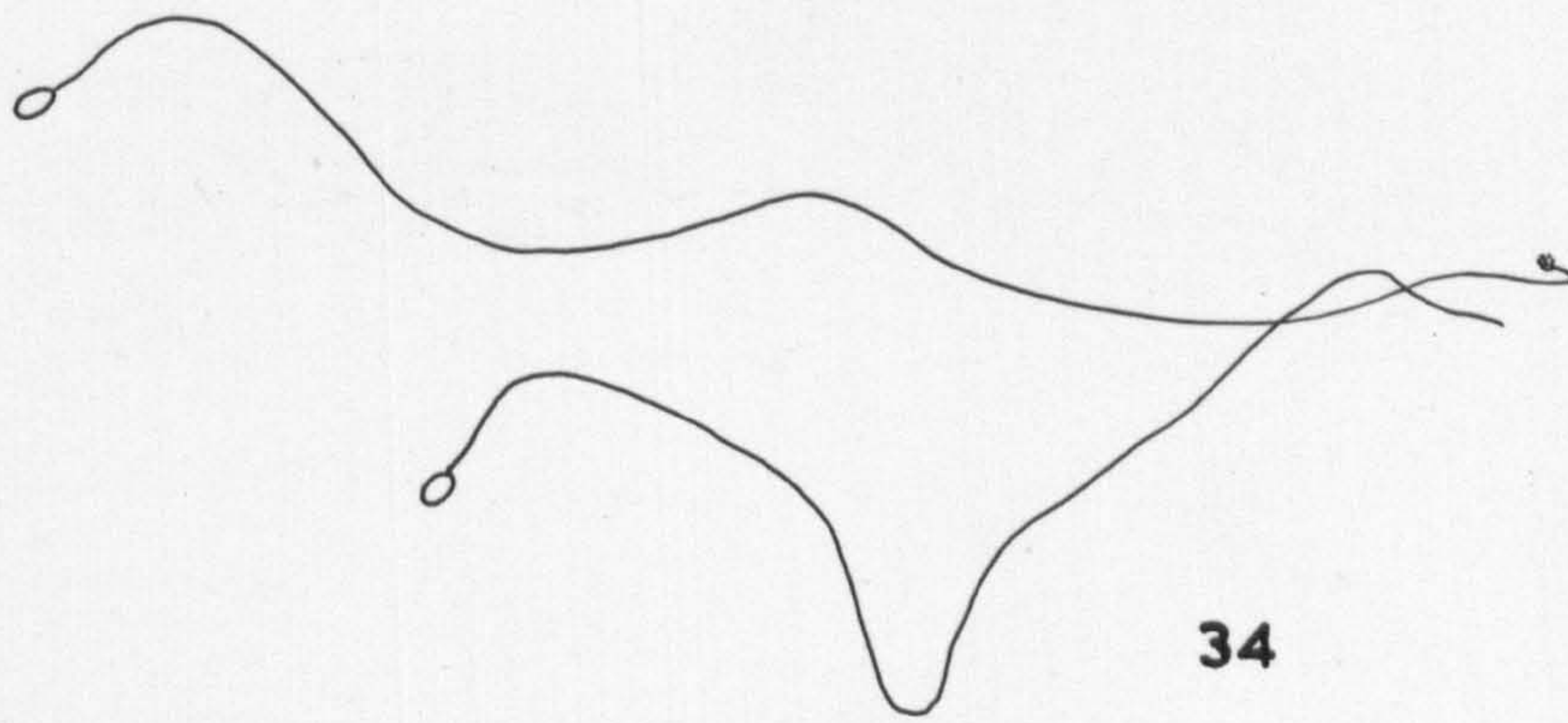
10μ.

32



33

10μ.



34

0 40μ.



addition to the oöcysts of T. garnhami , numerous spores of a microsporidian . These spores ( Fig. 32 ) examined in saline were ellipsoidal and showed no evidence of any vacuoles at the poles. The surface of the spores was smooth and they measured  $4.8 - 5.7 \mu$  by  $2.8 - 3.2 \mu$ . with an average value of  $5.4$  by  $3.0 \mu$ . Filament extrusion was easily accomplished by mechanical pressure on the cover-slip, and one of the spores thus treated ( Fig. 33 ), showed at the tip of a much-coiled filament, a spherical protoplasmic globule which was no doubt the sporoplasm. The majority of extruded filaments were extremely long and measured from  $160 - 195 \mu$ . ( Fig. 34 ). No other morphological details of the parasite were available as the only infected pupa had already been crushed and so no sections could be made. But the smear preparation stained in Giemsa stain showed no evidence of any pansporoblasts and it is therefore concluded that the parasite is a Nosema , although there is no absolute evidence for this. The fact that the spores are larger than those of Nosema whitei Weiser, 1953 (which measured  $4.5 - 5 \mu$  by  $2 \mu$ . ), and the much greater length of the polar filament ( which in N. whitei is only  $60 \mu$ . ), suggests that the present parasite is a new species and it is therefore named Nosema buckleyi n.sp.



### Summary

The various stages of a new genus of schizogregarine Triboliocystis garphami gen. et sp. nov. are described in detail. The main characters are- :

1. Two types of schizonts , micronuclear and macronuclear.
2. The products of the second schizogony are different to those of the first.
3. Gametocytes are uninuclear when they associate.
4. As a rule eight oöcysts are found within each gametocyst.
5. Oöcysts are navicular with rounded poles which are membranous . Each oöcyst contains eight sporozoites.

The parasite was found in the fat body of the larvae of a culture of Tribolium castaneum causing their death when infection was heavy. There was evidence to show that autoinfection takes place with this parasite.

The systematic position of T. garphami is discussed and a brief description is given of a new microsporidian Nosema buckleyi n.sp. found in a single pupa of Tribolium .

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SUBSIDIARY MATTER



# Acephaline Gregarines of the Ceylon Earthworm, *Pheretima peguana*

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*By*

**A. S. Dissanaïke**

*Department of Pathology, University of Ceylon*

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## Acephaline Gregarines of the Ceylon Earthworm, *Pheretima peguana*

By

A. S. DISSANAIKE

*Department of Pathology, University of Ceylon*

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(With Two Plates)

(MS. Received 30th January, 1953)

Hitherto the gregarine parasites in the seminal vesicles of the common Ceylon earthworm, *Pheretima peguana*, were believed to be species of *Monocystis*. This belief was based, no doubt, on the assumption that local earthworms are hosts to parasites similar to those found in the majority of related Indian earthworms. While the characteristic gametocyte and gametocyst stages closely resemble monocystid types, an examination of the trophozoites and oöcysts, revealed that the parasites in the Ceylon earthworms belong to an entirely different family, the *Stomatophoridae*, as proved beyond doubt by the truncated poles of the pseudonavicellae or oöcysts. The adult trophozoites examined in saline solutions were seen to be more or less cup-shaped with cytoplasmic papillae and filaments, and to belong to two species of a new genus, for which the name *Zeylanocystis* is proposed. It is proposed to name the two species *Zeylanocystis burti* and *Zeylanocystis fernandoi*, after my teachers, Mr. D. R. R. Burt and Professor Wilfred Fernando.

### Material and Methods

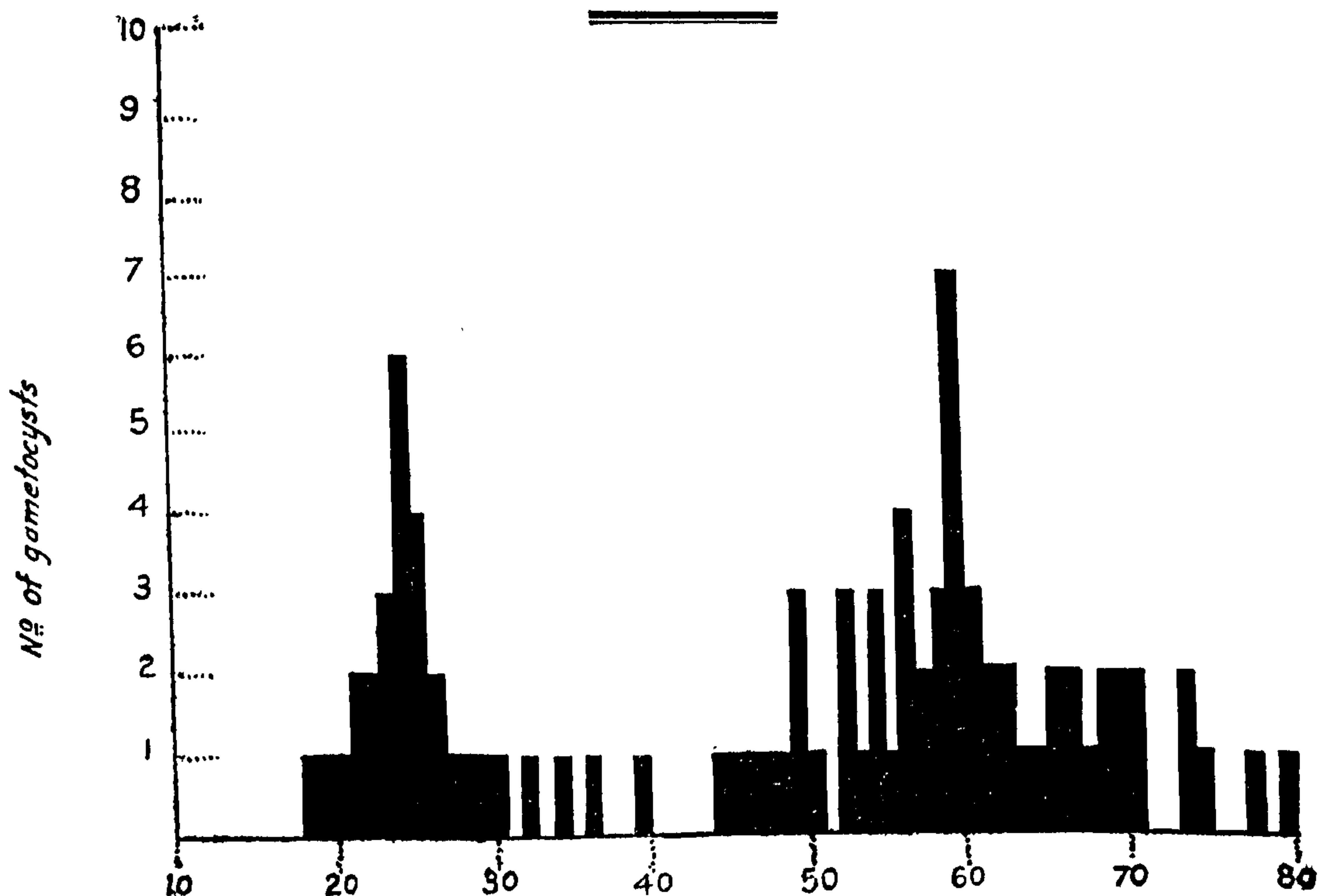
Measurements, and studies of the behaviour of the various stages of the parasite were carried out in vivo in Ringer's solution. A few broken cover-glasses placed between cover-glass and slide helped to avoid compression. Material for sections, and smears, were fixed in either Bouin's or Schaudinn's fluids. The following stains were used: Delafield's, Ehrlich's and Weigert's haematoxylin; Mallory's triple stain. The haematoxylin stains were counter-stained with eosin.

Gametocyst sizes were measured by taking two diameters at right angles to each other and taking the mean of these values as representing the diameter in each case. Measurements of trophozoites were made likewise across their rims.

A close examination of the measurements of trophozoites, gametocysts and oöcysts established two distinct sizes of the parasite, showing that there are two species, a small and large. It was found also that the smaller species may sometimes occur by itself in a worm, but that the larger is invariably found together with the smaller. The accompanying chart (Chart I), and Tables I, II, and III show quite clearly that there are two species.

It was found that while oöcyst sizes remain constant, whether measured in saline, smears or sections, those of trophozoites and gametocysts vary according to the preparation. For



CHART I

*Diameter of gametocyst in divisions on micrometer eyepiece*

instance, in smears, due to flattening, measurements are much greater than in saline ; while in sections, due to shrinkage, the values are smaller.

TABLE I  
*Trophozoites*

		<i>Diameter</i>	<i>Nuclei</i>
Ringer's	Z. fernandoi	35-46 $\mu$	—
	Z. burti	70-88 $\mu$	—
Smears	Z. fernandoi	35-56 $\mu$	8-10 $\mu$
	Z. burti	75-95 $\mu$	12-17 $\mu$
Sections	Z. fernandoi	—	—
	Z. burti	—	10.5-14 $\mu$

TABLE II

*Gametocysts*

*Average Diameters*

Ringer's	<i>Z. fernandoi</i>	63·2-122·5 $\mu$
	<i>Z. burti</i>	140-192·5 $\mu$
Smears	<i>Z. fernandoi</i>	70-140 $\mu$
	<i>Z. burti</i>	175-245 $\mu$
Sections	<i>Z. fernandoi</i>	—
	<i>Z. burti</i>	—

TABLE III

*Oöcysts*

*Length*

*Breadth*

Ringer's	<i>Z. fernandoi</i>	7 $\mu$	3·5 $\mu$
	<i>Z. burti</i>	14-14·7 $\mu$	—
Smears	<i>Z. fernandoi</i>	7 $\mu$	3·5 $\mu$
	<i>Z. burti</i>	14-14·35 $\mu$	7 $\mu$
Sections	<i>Z. fernandoi</i>	7 $\mu$	3·5 $\mu$
	<i>Z. burti</i>	14 $\mu$	7 $\mu$

Stages in Life-Cycle

**Trophozoites (Figs. 1-9)**

These are more or less flattened discs in the earlier stages, becoming more cup-shaped as they mature. The immature trophozoites are best described as saucer-shaped. From the edges of these discs a large number of symmetrically spaced mobile cytoplasmic papillae project. These filaments are equal in size when fully extended. When observed for some time in Ringer's solution, these papillae and filaments are seen to contract and to get absorbed into the general peripheral cytoplasm forming a thickened rim. The saucer-shaped nature of the trophozoites is readily observed by pressing the cover-glass down and noticing them as they roll about.

When examined in saline solutions the nuclei of trophozoites appear as clear circular areas at the centres of the discs, whether viewed from the convex surface ('posteriorly') or from the concave surface ('anteriorly'). In either view the cytoplasm appears granular, denser at the centre, with darker x-shaped areas which correspond, no doubt, to myonemes (miocite-Cognetti).



Sections of trophozoites show well-marked myonemes extending 'antero-posteriorly'. The nucleus is spherical with a well-marked, centrally placed nucleolus. An interesting feature is the presence of a more refringent spot within each nucleolus.

In some sections three to five immature trophozoites are seen piled one on top of the other like a pile of plates. (Fig. 9).

### Gametocytes, Gametocysts and Gametes (Figs. 10-15)

The trophozoites described above are flattened at earlier stages and later increase in thickness 'antero-posteriorly' until they finally become more or less spherical or ovoidal. They are now adult trophozoites (telotrophozoites-Cognetti) and are ready to associate in pairs and secrete a gametocyst. By now the cytoplasmic papillae and filaments have disappeared, and so have the myonemes.

Later stages of gametocytes and of gamete formation are typical of the acephaline gregarines and are shown in Figs. 10-15.

Another interesting feature is that the residual cytoplasm is seen to form a single, rounded mass at the centre of each gametocyst after gamete formation is complete.

Gametocysts in the larger species are more spherical than those in the smaller, which are ovoidal.

Gametes are isogametes. Zygotes are distinguished by their ovoid shape and by the fact that they are readily stained, unlike the later stage or oöcyst which is less easily stained on account of its resistant covering.

### Oöcysts (Fig. 16)

These are found to be characteristically navicular, containing, when mature, eight sporozoites (seen best in sections) and having similar poles that are truncated. They measure on an average  $7\mu$  by  $3.5\mu$  in the smaller species and  $14.5\mu$  by  $7\mu$  in the larger.

### Discussion

In the past the gregarine parasites in the seminal vesicles of *Pheretima peguana*, were wrongly assumed to be species of *Monocystis*. This was, no doubt, partly due to a failure to study details of the structure of trophozoites, and partly to the fact that the commonest acephalines in related Indian earthworms belonged to the genus *Monocystis*.

While *Zeylanocystis* gen. nov. has none of the features characteristic of the family *Monocystidae*, it has the typical characters of the family *Stomatophoridae* which are as follows:—

'Trophozoites spherical or cup-shaped with a sucker-like organ anteriorly; solitary; oöcysts navicular with truncated poles; in seminal vesicles of *Pheretima*'. (Kudo, 1946).

In *Zeylanocystis* the convex surface of the trophozoite could be regarded as representing the posterior end, and the concave surface as the anterior. The rim of cytoplasmic papillae and filaments, which is mobile, represents the anterior sucker-like organ.

It remains now to discuss the systematic position of *Zeylanocystis* within the family *Stomatophoridae*. Kudo describes seven genera in this family, viz., *Stomatophora*, *Astrocystella*, *Craterocystis*, *Beccaricystis*, *Choanocystis*, *Albertisella*, and *Choanocystoides*. But they all differ in various respects from *Zeylanocystis* except perhaps *Choanocystis*, *Choanocystoides* and *Albertisella* which resemble *Zeylanocystis* at least in shape. *Choanocystis* possesses a central

tentacle with cytoplasmic hairs, and this tentacle projects from the anterior concave surface. *Albertisella*, though very similar in appearance, especially in sections, lacks cytoplasmic hairs and papillae. *Chaenocystoides*, on the other hand, bears the closest resemblance to *Zeylanocystis*. It is more or less cup-shaped, with a mobile sucker like organ anteriorly, and carries cytoplasmic hairs. But it differs from *Zeylanocystis* in the following respects —

- (1) The cytoplasmic hairs or filaments in *Chaenocystoides* vary in length, whereas in *Zeylanocystis* they are all of equal length and symmetrically arranged.
- (2) There are no cytoplasmic papillae in *Chaenocystoides*.
- (3) The arrangement of myonemes is different. In *Chaenocystoides* they are confined almost entirely to the anterior end. In *Zeylanocystis* they extend antero-posteriorly.

The earthworms used in this study were collected in Colombo only, and cannot be regarded as representing those of the whole Island. Yet it is interesting to find that no species of *Monocystis* were found in local earthworms, whereas in India *Monocystis* is a common type, though other types such as *Apolocystis* have been described. Furthermore, the only members of the family *Stomatophoridae* reported so far from India are three species of *Stomatophora* (Bhatia, 1938).

A more detailed study of earthworms from all parts of the Island is necessary before any further conclusions could be drawn, but from the above it is clear that *Monocystis* is not found, at any rate in Colombo, and that the predominant species is a hitherto unknown member of the family *Stomatophoridae*, belonging to a new genus, *Zeylanocystis*, which is closely related to *Chaenocystoides* described by Cognetti (1925) from a Costa Rican earthworm, *Pheretima heteroclada*. A larger species *Zeylanocystis burti* has been found to co-exist with a smaller species *Zeylanocystis fernandoi*, in *Pheretima peguana* in Ceylon.

### Summary

1. Two species of Acephaline gregarines are common in *Pheretima peguana* in Ceylon.
2. These have been shown to belong to the family *Stomatophoridae* and to a new genus *Zeylanocystis*, which is closely related to the genus *Chaenocystoides*.
3. The smaller species *Zeylanocystis fernandoi* may occur alone, but the larger species *Zeylanocystis burti* is invariably found to co-exist with the smaller.

### Acknowledgments

The present work was started at the suggestion of Mr. D. R. R. Burt, former Professor of Zoology, University of Ceylon, to whom I am grateful. My thanks are also due to Professor Wilfred Fernando of the Department of Zoology, University of Ceylon, with whose help and guidance this work was completed, and to Dr. Hilary Cross of the Department of Zoology, University of Ceylon, for much invaluable help and encouragement given from the beginning of this work till it was ready for publication.

I must also thank Professor R. R. Kudo of the University of Illinois, for his critical reading of the manuscript, Dr. T. W. Wikremanayake, presently at Glasgow, for sending me most of the literature that was not available in Ceylon, and Mr. Scott Duckie for helping me with the translations.



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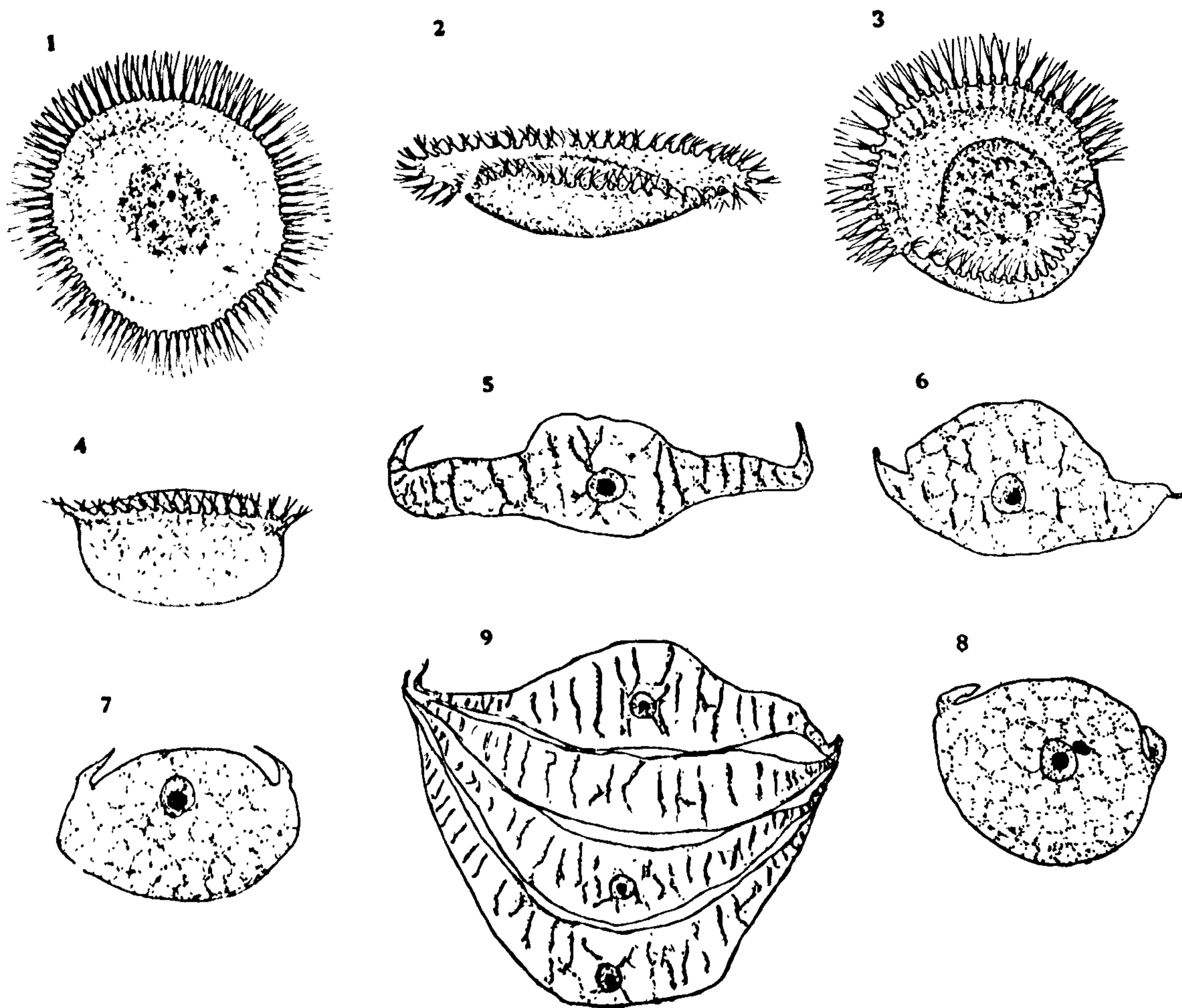
## EXPLANATION OF PLATES

## PLATE XXXVI

- FIG. 1—*Zeylanocystis burti*, trophozoite, posterior view (in saline).  $\times 375$ .  
 FIG. 2—Trophozoite, side view (in saline).  $\times 500$ .  
 FIG. 3—Trophozoite, anterior view (in saline).  $\times 375$ .  
 FIG. 4—Telotrophozoite, side view (in saline).  $\times 375$ .  
 FIG. 5—Trophozoite, L.S.  $\times 750$ .  
 FIG. 6—Trophozoite, later stage, L.S.  $\times 600$ .  
 FIGS. 7 and 8—Telotrophozoites, L.S.  $\times 600$ .  
 FIG. 9—Trophozoites, showing piled-up arrangement, L.S.  $\times 750$ .

## PLATE XXXVII

- FIG. 10—*Zeylanocystis burti*, gametocyte (in gametocyst) in section.  $\times 225$ .  
 FIG. 11—Gametocytes showing nuclei at periphery.  $\times 225$ .  
 FIG. 12—Gametocytes showing gamete formation.  $\times 225$ .  
 FIG. 13—Gametocyst with zygotes and residual cytoplasm.  $\times 225$ .  
 FIG. 14—Gametocyst with zygotes, later stage.  $\times 225$ .  
 FIG. 15—Gametocyst with oöcysts.  $\times 225$ .  
 FIG. 16—Oöcysts, T.S. and L.S.  $\times 2,500$ .



- FIG. 1—*Zeylanocystis burti*, trophozoite, posterior view (in saline).  $\times 375$ .  
 FIG. 2—Trophozoite, side view (in saline).  $\times 500$ .  
 FIG. 3—Trophozoite, anterior view (in saline).  $\times 375$ .  
 FIG. 4—Telotrophozoite, side view (in saline).  $\times 375$ .  
 FIG. 5—Trophozoite, L.S.  $\times 750$ .  
 FIG. 6—Trophozoite, later stage, L.S.  $\times 600$ .  
 FIGS. 7 and 8—Telotrophozoites, L.S.  $\times 600$ .  
 FIG. 9—Trophozoites, showing piled-up arrangement, L.S.  $\times 750$ .



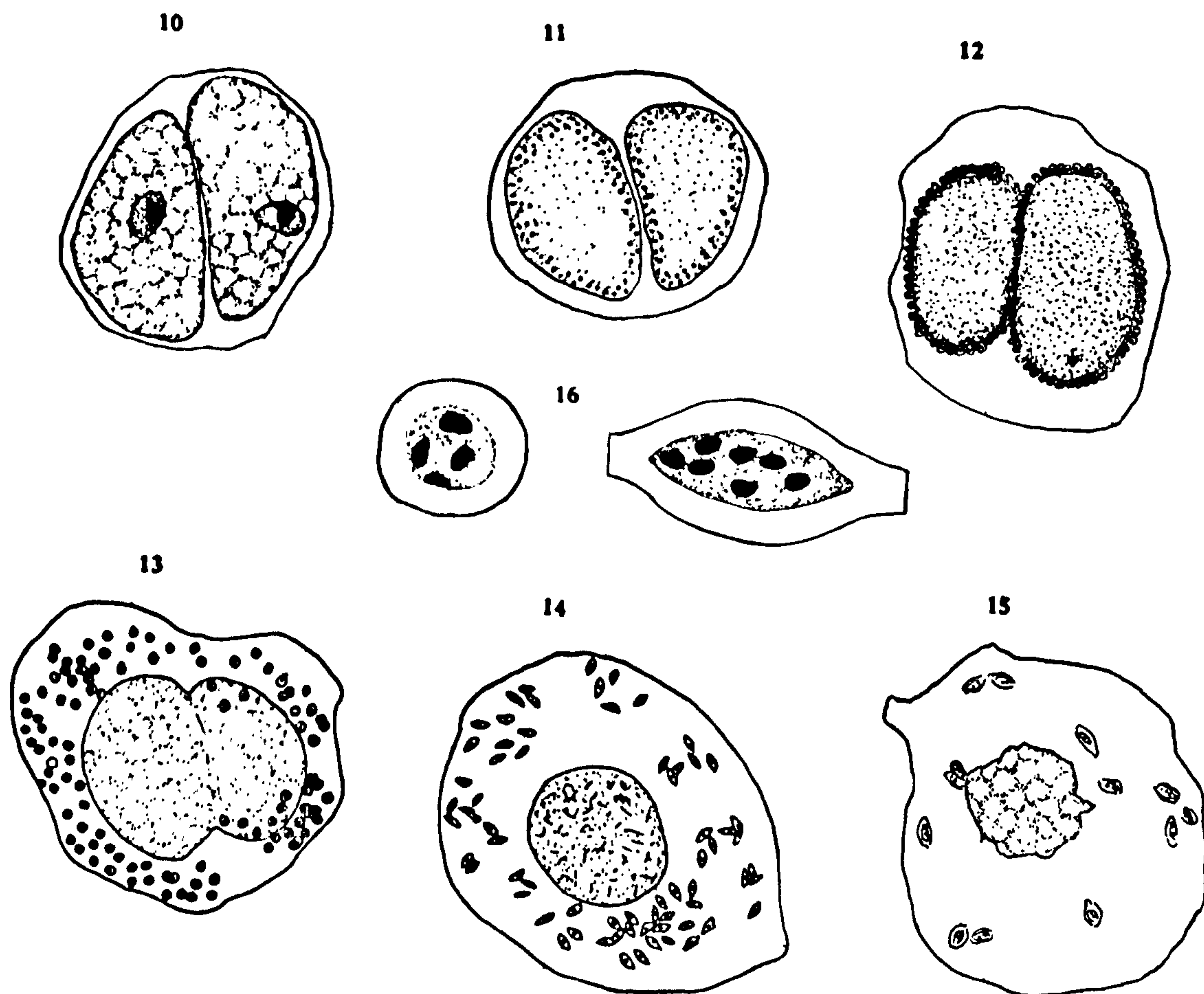


FIG. 10—*Zeylanocystis harti*, gametocytes (in gametocyst) in section.  $\times 225$ .  
 FIG. 11—Gametocytes showing nuclei at periphery.  $\times 225$ .  
 FIG. 12—Gametocytes showing gamete formation.  $\times 225$ .  
 FIG. 13—Gametocyst with zygotes and residual cytoplasm.  $\times 225$ .  
 FIG. 14—Gametocyst with zygotes, later stage.  $\times 225$ .  
 FIG. 15—Gametocyst with oöcysts.  $\times 225$ .  
 FIG. 16—Oöcysts, T.S. and L.S.  $\times 2,500$ .







# Enterobius Vermicularis in a Suppurating Cyst of the Female Breast

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*By*

**G. H. Cooray, Mangayarkarasi Yoganathan and  
A. S. Dissanaiké**

*(Department of Pathology, University of Ceylon)*

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## Enterobius Vermicularis in a Suppurating Cyst of the Female Breast.

By

G. H. COORAY, MANGAYARKARASI YOGANATHIAN AND  
A. S. DISSANAIKE

(Department of Pathology, University of Ceylon)

Although the adult *Enterobius vermicularis* has been reported to occur in unusual situations such as the soft tissues around the anus and rectum (Fitzwilliams, 1934), the kidneys and ureters (Symmers, 1950), the liver (Dieckmann, 1924, cited by Symmers), the spleen (Nathan, 1928, cited by Symmers), the female genital tract, the ovaries (Gill and Smith, 1952, Klee, 1920, Chiari, 1928, cited by Symmers, and Chomets, 1942), the nasal and oral mucosa (Proskauer, 1891, Chiari, 1889, and Pomper, 1877, cited by Symmers) the oesophagus and stomach (Brumpt, 1949) and beneath the mucous membrane of the intestines and rectum (Bijlmer, 1946), there has been no record of this parasite inhabiting the female breast. We describe such a case where an adult female was found in a suppurating cyst of the breast.

### Case Report

A female, aged 42 years, was admitted to a provincial hospital in Ceylon, with a history of a lump in the right breast of five days' duration. The lump was about the size of a pea when first noticed and grew much larger within the five days. There was no history of pain, itching or ulceration of the skin.

On examination, there was a cystic lump one inch in diameter in the lower and inner quadrant of the right breast. It was not connected with the nipple. The skin was slightly adherent to the lump and there was a distinct punctum. There was no tenderness over the lump, and no discharge from it or from the nipple. It resembled a sebaceous cyst. The lump was removed and sent to us for microscopic examination specially with regard to malignancy. For one month after the operation the wound which had broken down was discharging, and the tissue around the wound was indurated. The patient ran a swinging temperature for about two weeks after the operation.

The stools tested on three consecutive days showed no amoebae, cysts or helminth ova. There was no eosinophilia in the peripheral blood, the total leucocyte count being 7,200 /cu. mm. with a differential count of 69 per cent. Polymorphs and 31 per cent. Lymphocytes.

*Histology.* Beneath the epidermis which appeared normal there was a small abscess surrounded by cellular granulation tissue (Fig. 1). In this tissue there were numerous strands of keratin (Fig. 2) and adherent to the deep aspect of the granulation tissue mass there was a recent haemorrhage embedded in which were parts



of a nematode (Fig. 3). Several ova, both inside and outside the worm were also seen (Fig. 4). Closer examination of the worm proved that it was a mature female *Enterobius vermicularis* for the following reasons :—

1. The somatic musculature was of the Meromyarian type (Fig. 5).
2. The cuticle at one spot showed a characteristic lateral crest (Fig. 6).
3. The eggs had a double wall, were planoconvex in shape and on an average measured 52.9 by 20.6.

### Discussion

In a recent review of the pathology of oxyuriasis, Symmers (1950) mentions a large number of atypical sites in which *Enterobius vermicularis* had been reported and he discusses the pathological changes in the tissues evoked by the presence of the parasite. The present case where the parasite was found in a female breast is rather exceptional.

We have already given reasons for considering this worm to be a mature female *Enterobius vermicularis*. We also made serial sections of a mature female *Enterobius* obtained from the stools of a child and compared these with the parasite seen in the present case, and we have no doubt as to the identification of the nematode. It remains now to discuss the mode of entry of the worm, and the changes produced by it in the tissues.

Fulleborn (1929) claims that nematode larvae arriving in the lungs by the blood-stream may also pass by way of the pulmonary veins, left ventricle and the arteries, directly to the intestines and other organs of the body, but as threadworms do not undertake a lung journey, we can assume that the parasite in this case reached the present site from without. It may be assumed also that either an ovum, or larva or an adult worm had entered the breast tissue in this way. As it is unlikely that either of these would have penetrated intact skin, we consider that the most likely mode of entry was through the punctum which was observed clinically.

As threadworm ova and larvae are commonly found on the fingers of infected individuals, it is easy to imagine how one of these could have entered through this punctum; but it is difficult to understand how they could have developed into a fully mature worm in this situation. Since there were mature ova, within the section of the worm, and scattered amongst the adjacent tissues, it is reasonable to assume that the parasite was already a mature female when it entered through the punctum.

The absence of degenerative changes in the parasite which usually follow its death and of a granulomatous reaction around it, and the presence of viable ova scattered in the adjacent tissues, suggest that the nematode was alive at the time of the operation.

From the histological study it would appear that this patient had an epidermoid cyst of the breast, her attention being directed to it by the recent onset of suppuration. The parasite probably entered the sub-epidermal tissue through the punctum in the skin and its subsequent passage in the tissues caused a traumatic haemorrhage. Accidental contamination of the skin either by the patient's own infected fingers or from the peri anal skin of an infected child whom she may have carried most probably caused the nematode to be conveyed to this unusual site. Such a mode



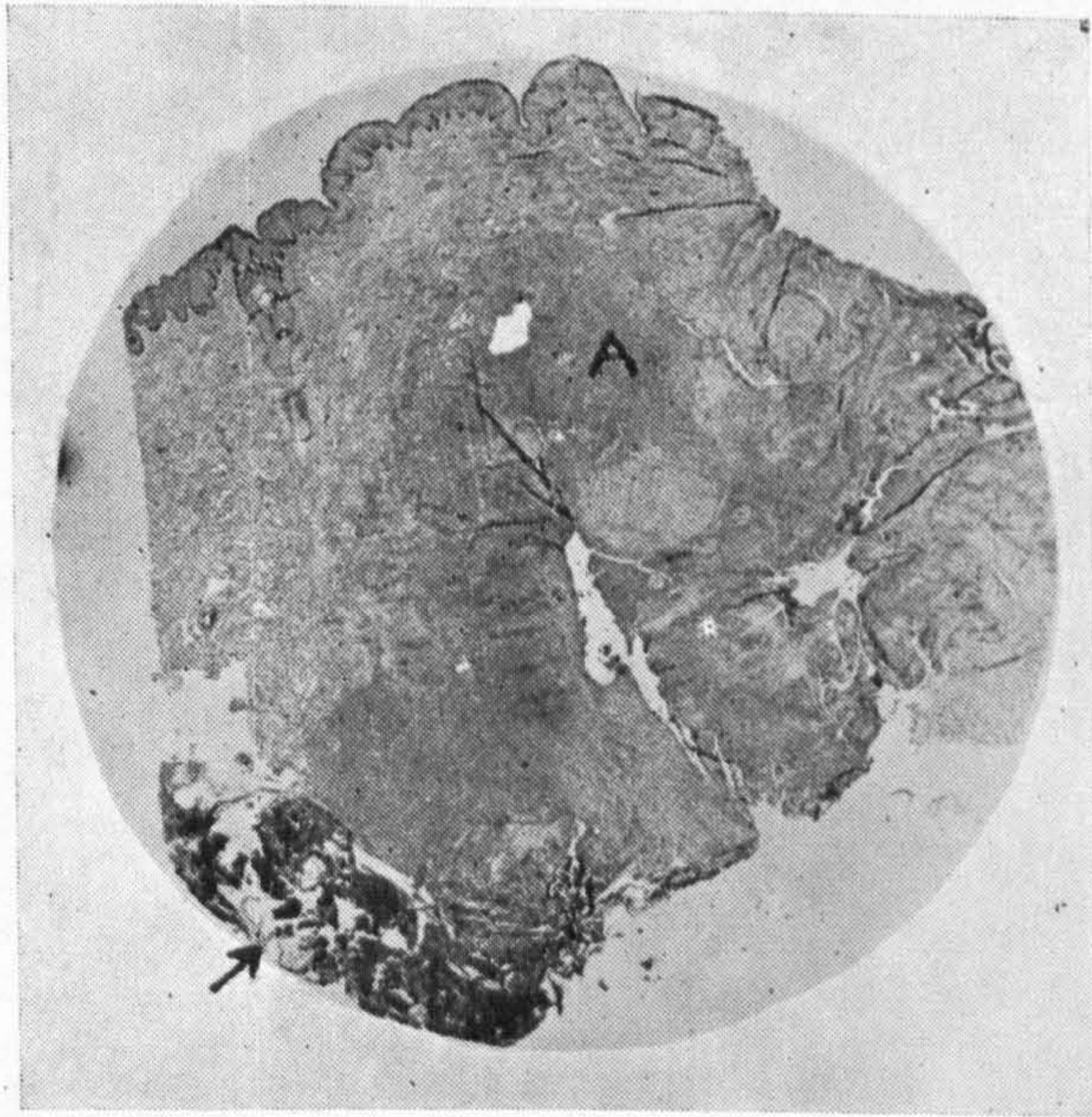


Fig. 1  
 Entire section (very low magnification) showing  
 abscess (A), and position of nematode in  
 bloodclot indicated by arrow.  
 H & E  $\times 6$ .

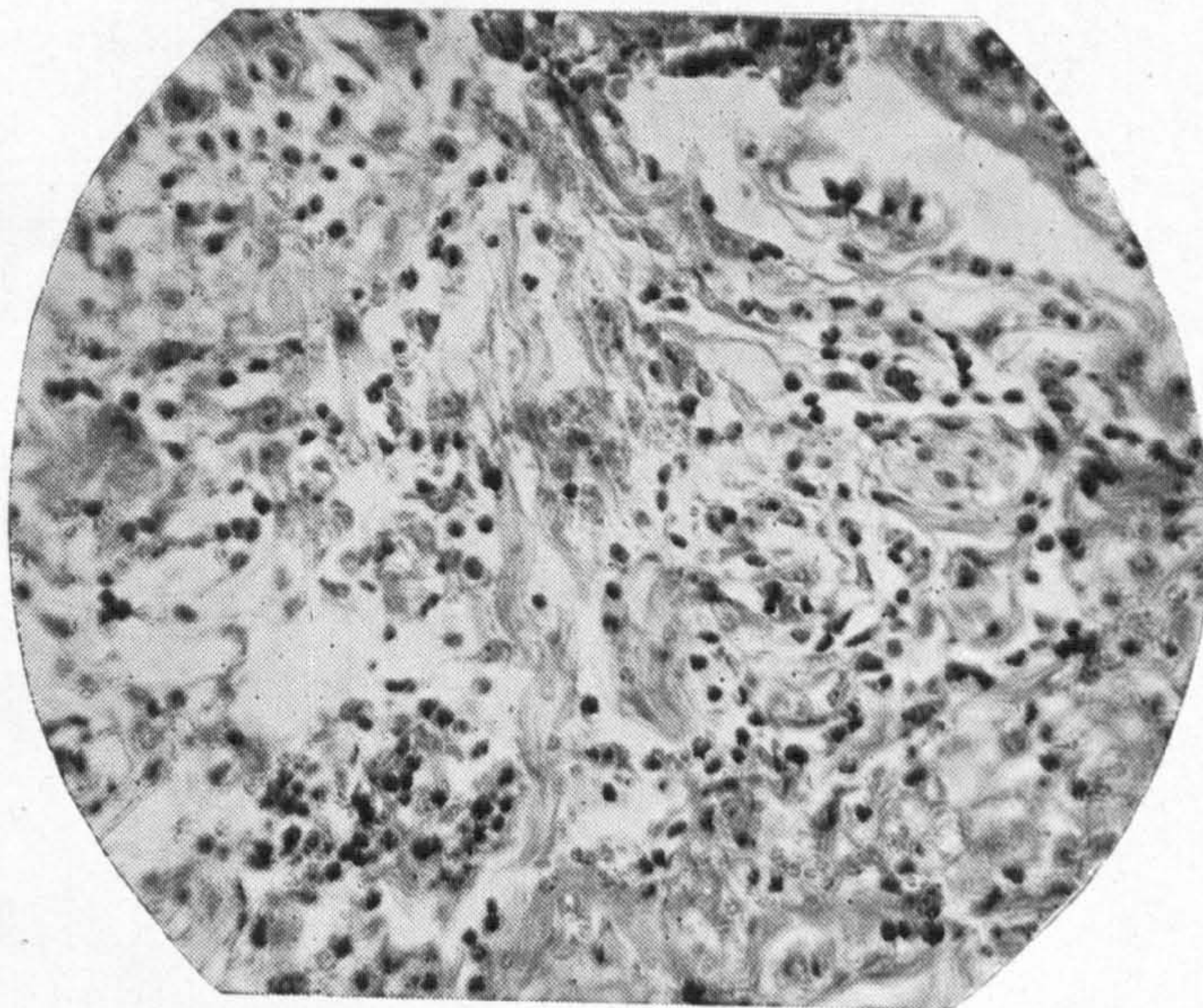


Fig. 2  
 Granulation tissue showing strands of keratin.  
 H & E  $\times 250$ .



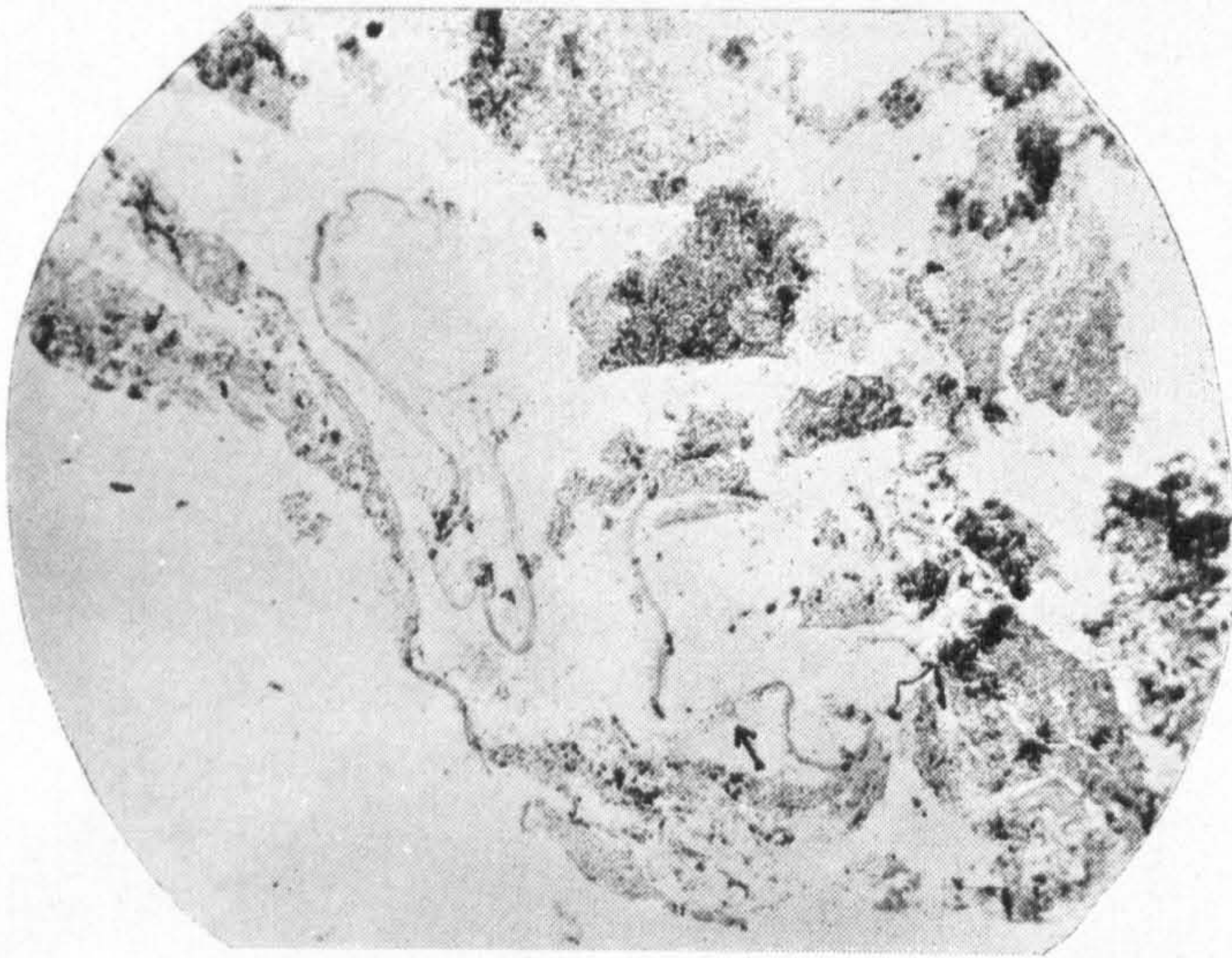


Fig. 3  
Bloodclot (higher magnification) showing parts of  
the nematode. Arrow points to position  
of lateral crest. H & E  $\times 60$ .

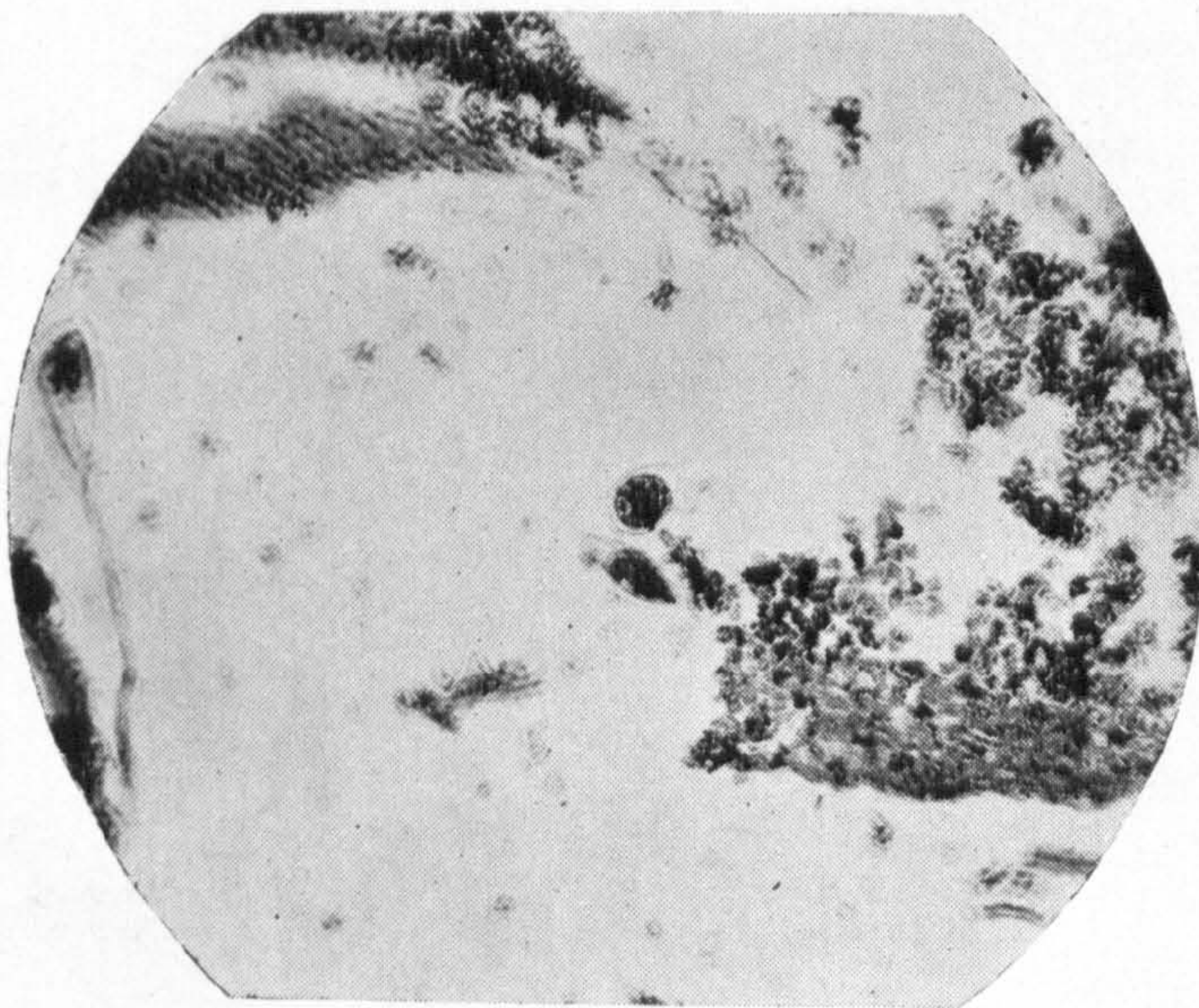


Fig. 4  
Two *Enterobius* ova. H & E  $\times 250$ .



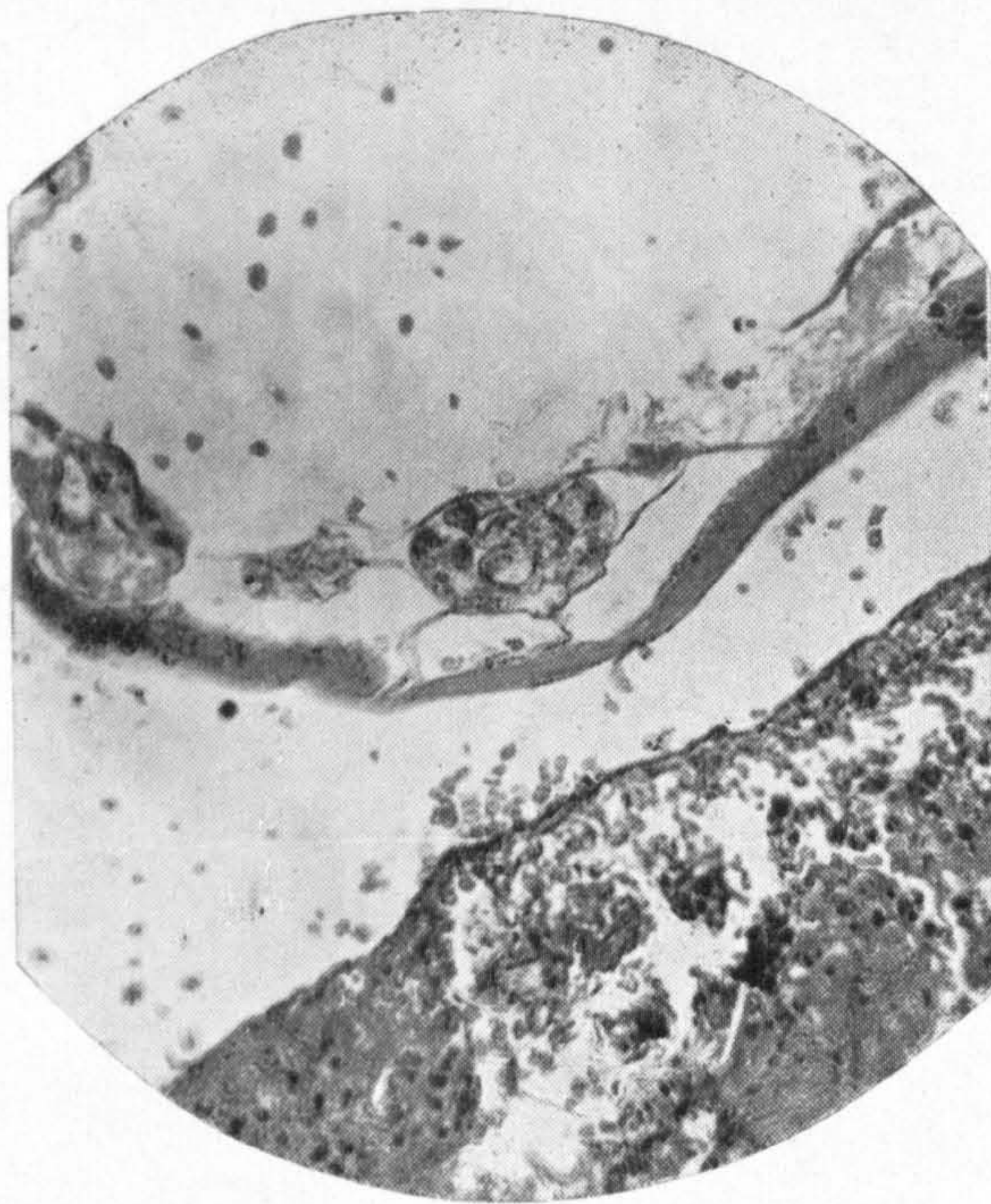


Fig. 5

Body wall of nematode showing simple Meromyarian musculature. H & E  $\times 250$ .



Fig. 6

Higher magnification of Fig. 3 showing lateral crest. H & E  $\times 250$ .



of entry through the skin is one of the suggestions put forward by Symmers (loc. cit.) to explain the presence of a gravid female *enterobius* in his case 3.

### Summary

1. A case is reported of the presence of an adult female *Enterobius vermicularis* in a suppurating cyst of the breast.
2. The typical granulomatous reaction of the tissues to the presence of this worm was not observed as the infection in this case was probably of very short duration.
3. The possible mode of entry and the identification of the worm are discussed.

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### Emergence of the Sporoplasm in *Nosema helminthorum*

THE mode of emergence of the infective sporoplasm from Microsporidian spores has baffled protozoologists for many years. According to earlier workers, the sporoplasm creeps out through the aperture from which the filament is extruded. Korke<sup>1</sup>, Ohshima<sup>2</sup>, Trager<sup>3</sup> and Gibbs<sup>4</sup> have described a protoplasmic globule at the tip of the extruded filament of the species studied by them. Ohshima and Gibbs thought that the filament pierces through the tissues, 'injecting' the sporoplasm into the host cell; filament extrusion, according to them, being either in a jack-in-the-box manner<sup>2</sup> or by eversion like the nematocyst of a coelenterate<sup>4</sup>. As Kudo<sup>5</sup> has remarked, these views ignore the delicate nature of the filament and the fact that the force required to inject the sporoplasm through a long tubular filament would injure it.

I have made some observations on *Nosema helminthorum* Moniez, 1887, which shed light on this problem and indicate the likely arrangement of structures within the mature spore. Spores from infected segments of *Moniezia expansa* left overnight in the refrigerator were examined in a drop of water covered with a cover-slip and lightly pressed. Several spores showed extruded filaments with globular masses attached to their tips; the corresponding spores were completely empty. This preparation was soon dry-fixed after gently removing the cover-slip, and stained in Giemsa stain. The resulting preparation showed more than twenty spores with extruded filaments having an irregular flattened mass at their tips (Fig. 1). These masses of protoplasm, which undoubtedly were the sporoplasms, were seen to be continuations of the filaments and each had a single nucleus also of irregular outline.

From these observations I conclude that the sporoplasm is normally attached to, and is probably a continuation of, the polar filament, which is not hollow. It would seem that inside the spore the coiled filament is surrounded by the sporoplasm in a girdle-like fashion. When the filament is extruded, it is 'whipped out' through a weak place in the anterior end of the spore in a 'jack-in-the-box' manner. The sporoplasm, being a continuation of the filament, is dragged out with it. In some sporas, extrusion is so rapid that the sporoplasm is left behind at the aperture of the spore; in other instances the sporoplasm breaks away from the tip of the